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CHARACTERIZATION OF METABOLIC DISEASE IN MOUSE MODELS OF ARSENIC EXPOSURE

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List of Abbreviations

ANOVA	Analysis of variance
ApoE	Apolipoprotein E
As	Arsenic
As^{3+}	Arsenite
AUC	Area under the curve
BAT	Brown adipose tissue
BMD	Bone mineral density
BMI	Body mass index
BPA	Bisphenol A
CD	Control diet
CNS	Central nervous system
CVD	Cardiovascular disease
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Diethylhexylphthalate
DEXA	Dual energy X-ray absorptiometry
DMA^{III}	Dimethylarsinous acid
DOHaD	Developmental origins of health and disease
EDC	Endocrine disrupting chemical
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide-1
Glut4	Glucose transporter, type 4
GSIS	Glucose-stimulated insulin secretion
GTT	Glucose tolerance test
HDL	High-density lipoprotein
HFD	High-fat diet
HOMA-IR	Homeostatic model assessment of insulin resistance
IHC	Immunohistochemistry
IP	Intraperitoneal
IRS-1	Insulin receptor substrate-1
ITT	Insulin tolerance test
LDL	Low-density lipoprotein
LXR	Liver X receptor
MCL	Maximum contamination level
MetS	Metabolic Syndrome
MMA^{III}	Monomethylarsonous acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NHANES	National Health and Nutrition Examination Survey
NTP	National Toxicology Program
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PDH	Pyruvate dehydrogenase

PFOS	Perfluorooctane sulfonate
PM	Particulate matter
POP	Persistent organic pollutant
PPB	Parts per billion
PPM	Parts per million
qRT-PCR	Quantitative real-time polymerase chain reaction
RER	Respiratory exchange ratio
rRNA	Ribosomal ribonucleic acid
Sbp2	Sec insertion sequence binding protein 2
SXR	Steroid and xenobiotic receptor
SYRINA	Systematic review and integrated assessment
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TG	Triglyceride
TZD	Thiazolidinedione
WAT	White adipose tissue
WHO	World Health Organization

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Abstract

The current epidemic of metabolic and cardiovascular disease is highlighted by the prevalence of type 2 diabetes mellitus (T2DM), a devastating disease that is growing at an astounding rate. Calorically-dense diets and physical inactivity, both of which have seen similarly dramatic increases in the past few decades, are only two amongst a myriad of risk factors contributing to metabolic dysfunction and T2DM. In recent years, more attention has turned toward other environmental factors that may be playing a role in exacerbating metabolic disease pathogenesis. Epidemiological studies have linked exposure to environmental pollutants known as endocrine disrupting chemicals (EDCs) with obesity, insulin resistance and T2DM. Additionally, numerous biochemical, cellular and animal models have shown that these EDCs have the capacity to alter normal hormonal signaling pathways both *in vitro* and *in vivo*. Exposure to arsenic (As), a toxic metalloid element, has been previously linked to chronic human disease states, including various cancers and metabolic diseases, in epidemiological studies. Recent studies estimate that over 100 million people worldwide are exposed to dangerously high levels of arsenic in their drinking water and through dietary sources.

As a result of these observations, the experiments described in this dissertation were conceived and executed in order to elucidate the mechanisms driving metabolic impairment resulting from arsenic exposure *in vivo*. In a mouse model characterizing chronic exposure to inorganic arsenic via drinking water, impairments in normal glucose metabolism are described. Collectively, the data from these experiments suggest that defective insulin secretion, as opposed to insulin resistance, drives the diabetogenic effects of arsenic exposure. Furthermore, dietary stress in the form of high-fat diet (HFD) feeding is shown to play an important role in arsenic-mediated metabolic dysfunction, and dietary manipulation proves to be a useful tool for studying

the mechanisms driving environmentally mediated disease. In order to impact public policy and bring about meaningful change in the way environmental chemical exposure is handled, it is necessary to provide data for how these exposures directly impact human health. The research described in this dissertation makes significant strides in characterizing chronic arsenic exposure as it relates to metabolic disease pathogenesis.

CHAPTER 1

INTRODUCTION TO ENVIRONMENTAL ENDOCRINE DISRUPTION AND DIABETES

Portions of this chapter has been adopted directly from the following:

[1] Kirkley, A.G., Sargis, R.M. "Environmental Endocrine Disruption of Energy Metabolism and Cardiovascular Risk." *Curr Diab Rep*. 2014; 14(6):494. doi: 10.1007/s11892-014-0494-0.

Section 1.1: Understanding Diabetes and Metabolic Disease as a Public Health Crisis

Section 1.1.1: Type 2 Diabetes Mellitus – Disease Pathogenesis and Risk Factors

The current metabolic disease epidemic presents a number of critical issues to public health. The incidence of chronic metabolic diseases, including type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS), has been increasing at an astounding rate in recent decades. T2DM exerts a tremendous individual and societal toll. Patients with diabetes have a risk of death approximately double that of their healthy peers, and T2DM remains the leading cause of kidney failure, blindness, and nontraumatic amputations [2]. Consequently, recent estimates suggest that total costs associated with diagnosed diabetes amount to a staggering $245 billion annually in the United States alone [3]. This massive economic and societal burden has increased at an alarming rate, with both disease incidence and associated costs exceeding that of even the most recent projections. As many as one-third of U.S. adults are expected to have T2DM by the year 2050 [4], and even optimal intervention strategies are projected to achieve only moderate success at reversing these trends at their current rates of growth [5]. Finally, these estimates do

not account for the global burden of the disease, which is expected to rise from 382 million to 592 million individuals worldwide by the year 2035 [6].

In addition to the clear association with microvascular complications such as retinopathy, neuropathy, and nephropathy; T2DM, type 1 diabetes, and other prediabetic conditions (e.g. impaired fasting glucose and glucose intolerance) are major risk factors for the development of macrovascular complications, including atherosclerosis, stroke, coronary artery disease, and peripheral vascular disease [7-9]. In fact, diabetes is considered an independent risk factor for cardiovascular disease (CVD) and mortality [10, 11]. T2DM is associated with several common CVD risk factors, including obesity, diabetic dyslipidemia, hyperglycemia, and insulin resistance. Importantly, the clustering of these metabolic risk factors in T2DM works in an additive fashion to promote vascular disease.

Although the genetic contributions to T2DM are substantial, environmental factors are often cited as the major drivers of risk for these chronic disease states. Increased energy intake, particularly consumption of calorically-dense foods common to the "Western diet", combined with an increasingly sedentary lifestyle clearly contribute to the current metabolic disease epidemic [12]. However, these factors alone fail to fully account for the magnitude of the metabolic disease epidemic.

Over the past few decades we have witnessed an important transformation in our knowledge of toxicity with the recognition that environmental pollutants have the capacity to modulate endocrine and metabolic signaling pathways, opening the door to a greater appreciation of the myriad factors contributing to the burgeoning global metabolic disease epidemic. Epidemiological studies support a role for a variety of organic and inorganic pollutants in the development of insulin resistance, obesity, and diabetes; and these studies are supported by

preclinical studies associating individual exposures with specific mechanisms of disease development. Voluntary and involuntary exposures to myriad synthetic chemicals are a common feature of modern society, and these exposures form the basis for many recent studies examining the links between environmental chemicals and the etiology of multiple chronic diseases [13-15].

Endocrine disrupting chemicals (EDCs) are a broad class of structurally diverse compounds that have the capacity to modulate endogenous hormonal signaling path- ways. These chemicals include industrial pollutants, waste products, pharmaceuticals, phytochemicals, pesticides, consumer products, and plastics; and they vary widely in both structure and mode of action. A wide variety of EDCs have been previously associated with an increased risk of T2DM and other metabolic disorders in both epidemiologic studies and experimental animal models [16-18]. Furthermore, these findings are supported by an increasing body of cell-based and biochemical studies demonstrating the capacity of these compounds to modulate insulin pro- duction in pancreatic β cells as well as insulin action in target tissues, which would be predicted to drive systemic metabolic dysfunction [19, 20].

The growing body of evidence suggesting that EDCs have the capacity to augment the development of metabolic diseases such as obesity and diabetes is compelling; however, less well studied is the role of these toxins in the pathogenesis of atherosclerosis and CVD. Only more recently have studies begun to examine links between environmental pollutants and macrovascular disease [21]. This data implicates several compounds that may accelerate the development of atherosclerosis through their effects on established risk factors common to both diabetes and CVD (e.g. obesity, dyslipidemia). Given the terrible burden of T2DM and macrovascular disease in modern society, understanding the links between environmental

pollutants and these disease states is critical for formulating effective prevention strategies and identifying novel therapeutic interventions.

Section 1.1.2: Genetic vs. Environmental Factors: Contributions to Diabetes Pathogenesis

Type 2 diabetes is a complex, multifactorial disease with both strong genetic and environmental components that underlie its progression. On the genetics side, numerous studies have identified genetic variants in humans that may increase susceptibility to developing T2DM [22]. Several studies have shown that different genetic variants in the form of single nucleotide polymorphisms (SNPs) are associated with T2DM [23]. However, the relevance of this genetic knowledge remains limited in clinical settings due to the fact that each individual genetic change is likely associated with a small effect size. Additionally, the lack of appropriate models for studying gene-gene and gene-environment interactions confounds the ability to make accurate and clinically useful risk predictions.

While environmental factors contributing to the development of obesity, the metabolic syndrome (MetS) and T2DM are well established (i.e. high-fat, calorically-dense diet intake), their interaction with the genetic mutations that contribute to disease pathogenesis are only beginning to be understood. Furthermore, the specific role of environmental toxins and endocrine disrupting chemicals (EDCs) in exacerbating genetically driven components of diabetes pathogenesis is thoroughly understudied. Understanding these core ideas is essential for understanding how data from basic science research can inform disease risk and clinical practice, particularly given current limitations.

Section 1.2: Endocrine Disrupting Chemicals (EDCs): Links to Human Disease

Section 1.2.1: Origin of EDCs as a Contributing Factor for Chronic Human Diseases

Of particular interest in the field of endocrine disruption is the possibility that susceptibility to adverse effects varies across the lifespan. The Developmental Origins of Health and Disease (DOHaD) hypothesis postulates that organisms have periods of unique sensitivity to environmental insults during development and that exposure during these sensitive windows predisposes to the development of disease later in life [24]. Furthermore, exposure during these periods may also result in heritable changes through epigenetic modifications that can promote development of disease in generations remote from the initial chemical exposure [25, 26]. In a recent study, the effects of perinatal BPA exposure were found to be dose-, sex- and time-dependent, further suggesting that the perinatal period is a critical window of EDC susceptibility [27]. Multiple studies have shown that exposure to BPA during pregnancy can alter metabolic homeostasis in both the mother and in her adult offspring [28-30]. Similarly, gestational and lactational exposure to perfluorooctane sulfonate (PFOS), a world-wide industrial pollutant once commonly used in stain and water repellents [31], was shown to impair glucose and lipid homeostasis in adult rats [32, 33]. Thus, consideration of not only the chemical but the timing of exposure across the lifespan is critical for assessing the effects of EDCs on energy metabolism.

Section 1.2.2: Observations and Studies Linking Endocrine Disruptors to Various Human Disease States

Epidemiologic studies have provided intriguing links between environmental contaminants and the development of diabetes and other metabolic diseases [18 22]. To date, the majority of studies connecting environmental exposures to diabetes and metabolic dysfunction

have focused on a narrow group of compounds for which exposure data is most complete. A recent meta-analysis by the National Toxicology Program (NTP) determined that there is sufficient evidence to support a positive association between T2DM and persistent organic pollutants (POPs) [34]. These diabetogenic POPs include the pesticide dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE), as well as pollutants from the dioxin and polychlorinated biphenyl (PCB) families [34]. A similar NTP analysis concluded that there was a potential connection between arsenic, a common groundwater contaminant, and diabetes [35]. Although was somewhat inconclusive, especially at lower levels of exposure, the data showed a relatively robust association at high levels of exposure. Urinary levels of bisphenol A (BPA), a monomer used in polycarbonate plastics that results in widespread exposure [36], has also been shown to be significantly associated with diabetes in the 2003-4 NHANES data set [37].

In addition to links between EDCs and diabetes per se, other studies have identified associations between various toxins and risk factors for diabetes such as obesity, the metabolic syndrome, and insulin resistance. Several POPs, including organochlorine pesticides and their metabolites (e.g. DDE) as well as various PCB congeners, positively associate with obesity, abdominal adiposity, and components of the metabolic syndrome [38, 39]. Phthalates are used in the plastics industry as well as in various consumer goods and medical devices [40], and phthalate metabolites have been associated with insulin resistance and abdominal obesity [41, 42]. Insulin resistance has also been shown to correlate with urinary concentrations of BPA [43] and serum dioxin levels [44]. Finally, air pollution has been implicated in metabolic derangements with exposure to particulate matter (PM) of either the 2.5 μm (PM2.5) [45] or 10 μm (PM10) [46] size correlating with either reduced insulin sensitivity or the incidence of

diabetes. Collectively, these studies suggest that a diverse array of environmental contaminants may play a central role in the pathophysiology of diabetes and its antecedent states in some individuals.

Section 1.2.3: Mechanisms of Metabolic Disruption by Environmental Pollutants

Taken together, epidemiologic data provides important evidence for the potential deleterious effects of EDCs; however, many of these studies are cross-sectional, making it difficult to draw conclusions regarding causality. The suggestions drawn from these studies are, however, supported by animal models of exposure that have examined the pathogenesis of diabetes. A number of compounds have been shown to promote glucose intolerance and frank hyperglycemia in animal models. These include organic toxins, such as the plasticizer diethylhexylphthalate (DEHP) [47], PCBs [48], and triphenyltin [49] as well as the inorganic contaminant arsenic [50]. In addition to overt disruption of glucose handling, several EDCs have been shown to promote hyperinsulinism and insulin resistance. Chronic exposure of mice to the PCB mixture Aroclor 1254 promoted insulin resistance and hyperinsulinemia [51]. In addition, BPA [27, 28], the polybrominated diphenyl ether (PBDE) flame retardants [52], POPs [53], atrazine [54], particulate air pollution [55], and arsenic [56] have all been shown to cause impairments in insulin action and glucose homeostasis. Interestingly, in one study, insulin resistance induced by exposure to PM2.5 air pollution was only observed in the presence of a high fat diet, suggesting potential synergy between EDCs and dietary risk factors for metabolic disease [55].

Disruption of nuclear hormone signaling may play an important role in mediating the negative effects of EDCs. Sex steroids play a critical role in lipoprotein metabolism, and

disruption of sex steroid signaling has been a central area of interest in the field of endocrine disruption. A variety of compounds have been shown to modulate estrogenic and/or androgenic signaling [57, 58], and some of these compounds may promote the development of an atherogenic lipid profile. Similarly, thyroid hormone is known to play a critical role in lipoprotein metabolism [59]. Disruption of thyroid hormone action has also been described for a number of different EDCs, including hydroxylated PCBs [60]. States of glucocorticoid excess (i.e. Cushing's Syndrome) are characterized by hypertension, dyslipidemia, and hyperglycemia, in addition to other abnormalities. Agents that promote glucocorticoid receptor signaling may therefore contribute to elevated CVD risk through multiple mechanisms. Tolylfluanid was shown to stimulate glucocorticoid action in adipose tissue [61]. These studies suggest a potential role for EDC disruption of glucocorticoid receptor signaling as a potential mediator of metabolic and vascular disease. Nuclear hormone receptors that play a role in pathways regulating energy and lipid metabolism (e.g. liver X receptor (LXR) and steroid and xenobiotic receptor (SXR)) may also be important sites of endocrine disruption [62].

In healthy individuals, glucose levels are maintained within a very tight range through an augmentation of insulin secretion from pancreatic β-cells in response to increases in insulin resistance [63]. Under conditions of significant and sustained insulin resistance, however, β-cells begin to lose their ability to adequately compensate at times of peak demand, and the individual transitions to a state of impaired glucose tolerance. Ultimately, the persistent β-cell stress results in insufficient insulin secretion even during periods of fasting, and the patient enters a state of frank T2DM. Thus, EDCs that impair β-cell insulin secretion or interfere with peripheral insulin action can promote the development of T2DM. The effects of various environmental contaminants on β-cell physiology and insulin action have been examined [64]. Several

compounds have been shown to disrupt β-cell function, promote β-cell death, or disrupt signal transduction pathways in β-cells. These include organic compounds such as TCDD [65], PCBs [66], BPA [67], and triphenyltin [68]. Inorganic compounds have also been shown to modulate β-cell function as well, including cadmium [69] and mercury [70]. Furthermore, arsenic in both its inorganic and methylated forms has been shown to disrupt β-cell function [71]. Interestingly, BPA [67]onso and PCBs [72] have been shown to augment insulin secretion; however, this may still reflect a deleterious disruption in energy homeostasis, possibly through insulin-induced insulin resistance.

In addition to those chemicals affecting insulin secretion, a number of compounds have been shown to antagonize cellular insulin action in a variety of experimental systems. TCDD [73], BPA [74], and DEHP [47] have all been shown to reduce insulin receptor levels in some studies, whereas the phenylsulfamide fungicide tolylfluanid [75], particulate matter [76], TCDD [73], and DEHP [47] have been shown to reduce levels of insulin receptor substrate-1 (IRS-1), a key intermediate in the insulin signaling cascade. Downstream of IRS-1, the insulin-stimulated activating phosphorylation of Akt (protein kinase B) has been shown to be attenuated by a host of environmental toxicants, including arsenic [77], particulate matter [78], PCB-77 [79], tolylfluanid [75], and BPA [28], whereas arsenic has also been shown to antagonize insulin action distal to Akt phosphorylation [80]. Finally, antagonism of cellular insulin action at the level of the facilitative glucose transporter, type 4 (GLUT-4) has been shown for DEHP [47], TCDD [73], and cadmium [81]. Thus, a host of environmental pollutants have the capacity to alter energy homeostasis through a variety of cellular mechanisms that are predicted to promote the development of diabetes and its associated complications.

In addition to direct effects on insulin production or insulin action, EDCs may augment the risk of T2DM indirectly by altering the expression of the various secreted factors that modulate global insulin sensitivity. For example, adipose tissue plays a critical role in energy metabolism through the secretion of a number of adipokines. Adiponectin is one such secretory product that promotes insulin sensitivity while also exerting anti-inflammatory effects and promoting β-cell function [82]. Environmentally relevant doses of BPA suppress adiponectin release from adipose tissue ex vivo [83]. In addition, cadmium [84], tributyltin [85], and particulate matter [86] have also been shown to reduce adiponectin expression and/or release. In addition to promoting dysglycemia, EDC-induced reductions in adiponectin may also accelerate atherosclerosis, as this adipokine appears to play an important protective role in the vasculature by suppressing foam cell formation and promoting macrophage cholesterol efflux [87]. In contrast, tumor necrosis factor-α (TNFα) and IL-6 induce insulin resistance and are increased by TCDD [73], PCB-77 [48], and particulate matter [78]. EDC-induced changes in these pro-inflammatory mediators may also play a role in enhancing the development of atherosclerosis [88].

Section 1.2.4: Clinical and Societal Significance of Studying EDCs

The current state of scientific evidence supports a potential role for EDCs in the pathogenesis of metabolic disease. Whether any specific individual can tie their disease to a particular exposure, however, is much more complex given the immense heterogeneity of chemicals, concentrations, combinations, timing, and durations of exposure. However, as improvements are made in identifying risk to specific individuals, current data may suggest how an individual's environmental exposure profile can influence both their metabolic disease

development and response to specific pharmacologic therapies. For example, SXR regulates expression of cytochrome P450 enzymes [89], and this nuclear receptor is disrupted by multiple EDCs, including DDT and nonylphenol [62]. This suggests that individuals with exposure to these EDCs may experience differential efficacy or enhanced side effects from drugs metabolized by these enzymes. Evidence that atrazine and organophosphate pesticides can potentiate the action of adenylate cyclase [90] may suggest enhanced efficacy for the use of agents with anti-glucagon effects in the treatment of diabetes in patients exposed to these agents.

Similarly, those with exposures contributing to the development of their diabetes may receive preferential benefit with either insulin replacement or an insulin-sensitizing agent (e.g. the thiazolidinedione (TZD) class of medications) depending on the mechanisms of action of the EDCs to which they are exposed. Given the fact that there are over 150,000 chemicals registered with the European Chemicals Agency [21], most exposure is polychemical, many chemicals have multiple mechanisms of action, dose-effect relationships are sometimes characterized as non-monotonic, and phenotypes are influenced by the timing of exposure; it may be impossible to achieve the level of granularity necessary to draw specific conclusions regarding the efficacy of any particular treatment. However, in instances of known, well-characterized exposures (e.g. occupational, recreational, accidental), our expanding knowledge base may ultimately provide us with the tools necessary to improve the care of specific patient groups.

Section 1.3: Arsenic and the Environment

Section 1.3.1: Arsenic as a Deleterious Toxin in Humans

Arsenic is a toxic metalloid element whose detrimental effects on human health have been well characterized for centuries. In acute settings, the toxicological effects of arsenic are

determined by the species (i.e. organified vs. inorganic forms) and oxidation state (i.e. 3+ valence, 5+ valence) of the exact chemical in question. Arsenate compounds, which are species containing arsenic in the 5+ valence state, are thought to uncouple the formation of adenosine-5-triphosphate (ATP) by replacing phosphate groups in key enzymatic reactions associated with cellular energy metabolism, including glycolysis [91]. Arsenite compounds, species containing arsenic in the 3+ valence state, on the other hand have been shown to readily react with thiol-containing entities such as glutathione and cysteine. Arsenite inhibition of essential enzymatic activities, including that of pyruvate dehydrogenase (PDH), may detrimentally inhibit cellular bioenergetics pathways [92].

Additionally, methylation of arsenic *in vivo* is thought to act primarily as a detoxification mechanism, but studies have shown that methylated arsenic compounds, including monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), intermediates in the metabolism of arsenic, can be equally toxic in certain settings. Currently, there is only limited information on the tissue levels of the organic arsenic species following exposure to inorganic arsenic.

In addition to acting detrimentally in acute exposure settings, arsenic compounds have long been studied for their effects in chronic disease settings. For example, arsenic is a known carcinogen in humans, and it has been linked to a number of different cancers in epidemiological studies, including kidney, bladder, skin and liver cancer [93-95]. Proposed mechanisms for arsenic and its carcinogenic effects include genotoxicity, disruption of cell proliferation, altered DNA repair and oxidative stress, among others [96].

Section 1.3.2: Understanding the Origins of Arsenical Compounds in the Environment

Human exposure to environmental arsenic species occurs from a number of different sources, and through numerous different routes of exposure. For the purposes of the research described in this dissertation, the focus will be on understanding exposure through arsenic-contaminated drinking water, the primary source by which humans are chronically exposed. Arsenic is present throughout the environment in a variety of different chemical species. To add to this, all of these species can be subjected to transformation via redox reactions, ionic substitutions, and biotransformation resulting from the evolution of detoxification strategies in living organisms [97]. All of these processes taken together result in the production of both inorganic and organic arsenic species, and these processes in turn have substantial implications for determining the degree of arsenic mediated disruption.

Past studies have shown that dietary modifications can significantly enhance, alleviate, and in some cases even reverse, phenotypes seen under normal nutrition[98-100]. This is of particular interest when examining environmental arsenic exposure because groundwater exposure disproportionally affects populations in rural areas with limited access to uncontaminated groundwater, especially in the developing world [101]. Even in the United States, it is estimated that people using unregulated well water sources are exposed to levels significantly higher than the threshold set by EPA regulations (10 ug/L) [102]. Rural areas in developing nations have some of the highest levels of groundwater arsenic contamination in the world [103]. For example, in rural regions of the Mekong delta in Cambodia, total groundwater arsenic measurements averaged 217 ug/L, with the highest individual read being 1610 ug/L [101].

While contaminated drinking water undoubtedly acts as the largest source through which humans are chronically exposed to arsenic, recent research has revealed that dietary exposure can also contribute to overall exposure levels. Different food source vary greatly in their background arsenic concentration, but rice and seafood are two of the most heavily scrutinized sources of dietary arsenic [104]. Importantly, bioavailability differs widely depending upon the source of arsenic exposure. Further work is necessary in order to understand how different dietary arsenic sources affect uptake, detoxification and elimination of potentially deleterious arsenic metabolites.

Section 1.3.3: Strategies Employed to Mitigate the Deleterious Effects of Arsenic Exposure

Given that preventative strategies are preferable to curative strategies in disease settings, substantial work has been done in order to mitigate human exposure to arsenic-contaminated drinking water. In the United States, the EPA has enacted the World Health Organization's (WHO) recommended safe maximum contamination level (MCL) limit for arsenic in drinking water at 10 ug/L, or 10 parts per billion (ppb). However, other regions with more limited regulatory oversight have relied heavily on humanitarian efforts to assist in the identification and remediation of heavily contaminated drinking water sources. One strategy that will necessarily be employed to assist in this process involved the coordinated efforts of local community members to identify contaminated water sources and educate other members as to the location of these sources and the associated risks [105]. Methods that can reliably and affordably detect arsenic contamination out in the field in real-time are essential for raising awareness and for initiating the process of identifying water sources of high- vs. acceptably low-arsenic concentrations [106]. Even with all of these efforts in place to create and implement prophylactic

solutions, millions of people are still at risk for chronic arsenic exposure, and it is essential for clinicians to develop therapeutic strategies for dealing with the myriad chronic disease states that may develop as a result.

Section 1.4: Research Studies Linking Arsenic to Disease Pathogenesis

Section 1.4.1: Epidemiological Studies Linking Arsenic Exposure to Disease in Humans

Epidemiological literature has shown correlations between chronic arsenic exposure and insulin resistance, as well as chronic hyperglycemia and T2DM in certain populations [107-109]. Not surprisingly, due to the challenges posed by large-scale population studies, this data collectively has been deemed inconclusive to establish causality in populations chronically exposed to low-dose arsenic [35]. However, the associations between arsenic exposure and metabolic disease remain strong, particularly in countries such as Bangladesh and Taiwan [110-112]. Importantly, because properly designed case-control experiments cannot be performed using human subjects, it is necessary to use experimental models to recapitulate conditions mimicking environmental arsenic exposure the laboratory. Establishing a robust mechanistic basis for the diabetogenic effects of arsenic is essential for moving the field forward, and as such, this dissertation and the experiments described within will in part address these important challenges.

Section 1.4.2: Cellular Models of *in vitro* Arsenic Exposure

Studies at the population and animal levels have provided insight into the potential role of arsenic in the pathogenesis of diabetes and metabolic disease; however, they fail to fully characterize the molecular mechanisms by which arsenic exerts its deleterious effects. In order to

identify pathophysiological pathways and identify potential therapeutic targets, several studies have aimed to identify the molecular mechanisms responsible for diabetogenic environmental toxicants. These studies show that arsenic, which has been implicated in the pathogenesis of T2DM *in vivo*, can actually modulate important cellular signaling processes involved in insulin production and secretion, insulin action, and other essential pathways that control glucose homeostasis [77, 113, 114].

In order to understand how arsenic disrupts metabolic processes from a mechanistic standpoint, many researchers have used *in vitro* biochemical and cell-based models in order to experimentally determine outcomes [103]. Understandably, given the role of metabolism in determining acute toxicity, experiments looking at arsenic's effects *in vitro* have focused on cell types that comprise key metabolic organs and tissues. In studies using cultured differentiated 3T3-L1 cells, a model for the adipocyte, researchers have shown that trivalent metabolites of inorganic arsenic had the ability to inhibit insulin signaling processes and insulin-stimulated glucose uptake [115]. In C3H 10T1/2 cells, another model of adipocyte derived from murine cells, arsenite exposure was able to inhibit differentiation *in vitro* [116].

In studies looking at the effects of arsenic on β-cell insulin secretion, researchers found that exposure of INS-1(832/13) cells to low levels of arsenite led to decreased glucose-stimulated insulin secretion in a dose- and time-dependent manner [114]. Based on data showing intracellular glutathione and intracellular H_2O_2 scavenging activity was dose dependently increased by exposure, the authors concluded that arsenic provoked an adaptive oxidative stress response that acted to increase antioxidant levels, thereby dampening ROS signaling crucial to the process of glucose-stimulated insulin secretion.

Numerous studies have utilized HepG2 human hepatoma cells in order to look at the effects of arsenic exposure in a hepatocyte model [117-119]. One group showed that exposure of HepG2 cells to environmentally relevant levels of arsenite resulted in elevated CRP expression and secretion [118]. Taken together, the numerous cellular models used to characterize mechanisms stemming from acute arsenic exposure *in vitro* provide important points at which to begin the search for relevant mechanisms involved in *in vivo* arsenic metabolism and subsequent metabolic disruption.

Section 1.4.3: Using Animal Models to Study Arsenic Exposure *in vivo*

Animal models are essential for studying metabolic processes in an integrated, physiological context. Past studies have utilized rodents, including mice and rats, in order to assess outcomes related to diabetes and metabolic disease development. In order to model the most relevant route of exposure seen in humans, most studies have utilized a paradigm where by rodents are exposed via drinking water that has been spiked with some concentration of inorganic arsenic compound [120, 121]. Using weanling mice, researchers have demonstrated that chronic exposure to arsenic induces significant impairments in glucose tolerance [122]. Prior studies have been instrumental in establishing dosage ranges for exposure, and have balanced observed decreases in water intake noted in multiple studies with the need to deliver a maximally high but consistent intake of arsenic [120].

However, not all studies have used this paradigm where arsenic is ingested continuously via *ad libitum* access to contaminated drinking water. For example, arsenic exposure in male Wistar rats was shown to induce diabetic-like conditions and increase markers of oxidative stress in the pancreas and the liver after only 4 weeks using an oral administration protocol [123].

Whether one model or another most accurately recapitulates the human exposure process is difficult to discern based on data currently available. It is important to remember that humans being exposed to arsenic in drinking water are often exposed to doses at least one order of magnitude or more lower than doses typically utilized in animal models [124].

Section 1.5: Outstanding Questions Concerning the Mechanistic Basis by which Arsenic Disrupts Normal Metabolic Processes *in vivo*

Despite the great strides that have been made in this field, there remain a substantial number of important questions that must be answered in order for researchers to more adequately address arsenic-related metabolic dysfunction. Past studies are characterized by inconsistency of metabolic phenotypes that are seemingly dependent upon any number of different experimental criteria, and the conflicting results produced from these studies only act to confuse the interpretation of data with respect to environmental exposure in humans. These variable factors include the strain and genetic background of rodents used, the length and route of exposure, the developmental window at which exposure is initiated, the dosage used (ranging from 10 ppb up to 50 ppm), choice of diet background, and housing environment, amongst others [125-129]. Studies up to this point have also failed to provide a complete analysis of whole-body energy metabolism as it relates to energy intake and utilization. Similarly, no studies characterizing energy expenditure or circadian rhythmicity of metabolic or behavioral parameters at the organismal level have been completed. Analyzing multiple metabolic tissue types within the same study is necessary for understanding how the different tissues act and respond together in a concerted fashion when exposed to arsenic chronically, something that has been commonly overlooked in many experimental designs. And while many studies have looked at glucose

homeostasis and related parameters in exposed animals, limited analyses of insulin dynamics in the context of deteriorating glucose metabolism have been performed. Finally, mouse models of arsenic exposure have failed to address disruptions to normal insulin secretion from the perspective of analyzing pancreatic islet physiology or morphology. By addressing concerns outlined in this section, the experiments described in this dissertation collectively make significant advances towards a more comprehensive phenotypic picture of *in vivo* outcomes following chronic arsenic exposure.

Section 1.6: Goals of this Research Dissertation

By unveiling the molecular mechanisms responsible for metabolic disruption resulting from arsenic exposure, the ultimate goal of this research is to not only learn more about how arsenic acts mechanistically as an endocrine disruptor, but also gain valuable knowledge about how exposure in mice alters metabolic parameters relevant for human disease. The goal of the experiments outlined in this dissertation is to link novel mechanisms of disruption with controlled *in vivo* exposure in order to discover points at which intervention may prove to be highly beneficial. I believe that this knowledge will significantly advance the field of endocrine disruptors by providing a complete model for how environmental contaminants acting chronically can be deleterious to metabolic health in a significant way.

For compounds such as arsenic, where exposure is more prevalent in less developed areas of the world, there exists an issue of social justice for the protection of exposed populations. In order to impact public policy and bring about meaningful change in the way environmental chemical exposure is handled, it is necessary to provide data for how exposure directly impacts human health. The execution of experiments outlined in this dissertation will make significant

strides towards linking *in vivo* chemical exposures to metabolic disease pathogenesis, and will therefore provide answers for some of the most important questions regarding environmental health threats.

CHAPTER 2

METABOLIC CHARACTERIZATION OF A MOUSE MODEL OF CHRONIC ARSENIC EXPOSURE

This chapter is verbatim with the exception of figure numbering from:

*Kirkley AG, Carmean CM, Ruiz D, et al. (2017) "Arsenic Exposure Induces Glucose Intolerance and Alters Global Energy Metabolism." *In revision for publication at the time of submission of this dissertation

Section 2.1: Abstract

The contribution of environmental pollutants acting as endocrine disrupting chemicals (EDCs) has recently been recognized as a potential contributor to the pathogenesis of metabolic disease. One such pollutant, arsenic, contaminates the drinking water of approximately 100 million people globally, and has been associated with insulin resistance and diabetes in epidemiological studies. Despite these clinical observations, the precise metabolic derangements induced by arsenic remain poorly characterized. In the present study, the impact of arsenic exposure on metabolic outcomes *in vivo* was examined. 8-week old male C57BL/6J mice were exposed to inorganic arsenite in their drinking water at a concentration of 50 mg/L for 8 weeks. Glucose metabolism was assessed via *in vivo* metabolic testing, and feeding behavior was analyzed using indirect calorimetry in metabolic cages. Pancreatic islet composition was assessed via immunofluorescence microscopy. Arsenic exposed mice exhibited markedly impaired glucose tolerance as compared to controls; however, no difference in peripheral insulin resistance was noted between the groups. Insulin secretion measured during glucose tolerance

tests was inappropriately normal given the rise in blood glucose observed in arsenic exposed mice. In metabolic cage analyses, arsenic exposure altered the normal diurnal rhythm of food intake and energy metabolism. Despite decreased insulin secretion, pancreatic β-cell, α-cell, and δ-cell mass were not altered. Taken together, these data suggest that arsenic exposure impairs normal glucose tolerance through a functional impairment in β-cells, as opposed to peripheral insulin resistance. Further elucidation of the mechanisms underlying the observed behavioral and β-cell-specific dysfunctions may inform future intervention strategies.

Section 2.2: Background and Introduction

Type 2 diabetes mellitus (T2DM) poses a growing burden to both individuals and healthcare systems worldwide [6]. Current estimates place the annual cost of diabetes at $245 billion in the United States alone [3]. Because diabetes is the leading cause of adult blindness, kidney failure, and non-traumatic amputations as well as a potent driver of cardiovascular disease [6, 130], understanding the factors that promote diabetes pathogenesis is crucially important. Sedentary lifestyles and unhealthy, destructive dietary consumption patterns are undoubted drivers of diabetes risk; however, these factors are not the only environmental threats to metabolic health [63]. An area of increasing interest in recent years is the contributions of environmental pollutants acting as endocrine disrupting chemicals (EDCs) to diabetes risk [131]. The increasing body of evidence linking various pollutants to diabetes mandates investigation into the mechanisms of how EDCs alter normal energy physiology [132].

Arsenic is one such EDC that is listed by the World Health Organization (WHO) as a top ten environmental contaminant of public health concern. Approximately 100 million people worldwide are chronically exposed to unsafe levels of arsenic in their drinking water [124].

Populations in areas of endemic arsenic contamination (e.g. Bangladesh, Taiwan) are exposed to arsenic drinking water levels that exceed the WHO-recommended upper limit (10 ug/L) by an order of magnitude or more [107, 133]. A recent 2012 meta-analysis sponsored by the National Toxicology Program (NTP) concluded that there exists limited to sufficient support for an association between arsenic and diabetes in populations with relatively high exposure levels (≥ 150 ug/L in drinking water) [35]. Since the publication of this report, additional epidemiological studies have provided further evidence to support links between arsenic exposure and diabetes risk [134]. Importantly, the conclusion of the NTP report highlighted the need for greater understanding of the mechanisms linking arsenic exposure to metabolic dysfunction, a finding supported by a recent assessment of research gaps in the field [97].

I*n vitro* and animal models have previously been used to understand the multitude of negative effects arising from arsenic exposure [135]. *In vitro* studies using adipocyte cell lines have demonstrated that low-dose arsenite (As^{3+}) inhibits responsiveness of insulin-signaling intermediates [77], suggesting that arsenic may disrupt glucose homeostasis by interfering with insulin action in its target tissues. Additionally, *in vitro* studies employing β-cell model systems have suggested that arsenic may attenuate insulin secretion [114]. Animal models of exposure have shown that rodents exposed to arsenic through drinking water exhibit impaired glucose homeostasis [120, 122, 136]. However, the specific mechanisms by which arsenic exposure exerts these effects *in vivo* remain incompletely understood.

The present study was conducted to illuminate the relative contribution of arsenic's effects on insulin action versus insulin secretion in whole-body glucose homeostasis. Chronic, sub-toxic arsenite ingestion in adult male mice exerted substantial diabetogenic effects, including impaired glucose metabolism; however, systemic insulin action was unaffected. Instead, glucose-

stimulated insulin release was attenuated, despite preservation of pancreatic islet endocrine cells, suggesting that arsenic contributes to diabetes pathogenesis primarily through functional impairments of insulin release as opposed to β-cell destruction.

Section 2.3: Materials and Methods

Section 2.3.1: Animal Care and Arsenic Exposure

Seven- to eight-week-old male C57BL6/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in pairs under 12-hour light/dark cycles at 22.2 ± 1.1°C. All animals received an *ad libitum* chow diet (Teklad Global Diet 2018; Envigo, Madison, WI). The arsenic exposed group was provided reverse-osmosis-purified bottled drinking water supplemented with 50 mg/L sodium arsenite (As^{3+}) (Sigma Aldrich, St. Louis, MO). The control group received the same water without arsenite supplementation. Body weight, food consumption, and water intake were measured weekly throughout the studies. Animals were treated humanely in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Section 2.3.2: Tissue Harvest and Preparation

After 8 weeks of exposure, animals were fasted for 5-6 hours and then euthanized using isoflurane anesthesia followed by exsanguination via cardiac puncture. Tissues of interest were dissected, weighed, and flash frozen in liquid nitrogen. Tissue samples were stored at -80°C until the time of processing.

Section 2.3.3: Intraperitoneal Glucose Tolerance Tests (IP-GTTs)

Following 8 weeks of exposure, mice were fasted for 6 hours, at which point baseline fasting blood glucose readings were obtained from all mice via tail vein sampling after application of local anesthetic (2% viscous lidocaine, Water-Jel, Carlstadt, NJ). Dextrose was injected intraperitoneally at a concentration of 2 g/kg body weight. Blood glucose levels were measured at 10, 20, 30, 40, 60, 90, and 120 minutes following injection using a Freestyle Lite glucometer (Abbott Laboratories, Abbott Park, IL). Blood samples were collected from the tail vein into heparinized microtainer tubes (Sarstedt, Numbrecht, Germany) at 0, 10, 30, and 60 minutes for determination of insulin concentrations. Tubes were placed immediately on ice, centrifuged at 1500 x g for 15 minutes at 4°C, and plasma was collected. Plasma insulin concentrations were measured using the Mouse Ultrasensitive Insulin ELISA kit according to the manufacturer's instructions (ALPCO, Salem, NH). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using fasting blood glucose and fasting plasma insulin levels as previously described [137].

Section 2.3.4: Intraperitoneal Insulin Tolerance Tests (IP-ITTs)

Following 8 weeks of exposure, mice were fasted for 3 hours, and fasting blood glucose levels were measured via tail vein sampling after application of local anesthetic. Mice were then injected intraperitoneally with Humalog insulin (0.5 U/kg body weight; Eli Lilly, Indianapolis, IN). Serial blood glucose readings were taken at 15, 30, 45, 60, 90, and 120 minutes following injection.

Section 2.3.5: Pancreatic Histology and Immunohistochemistry (IHC)

At the time of terminal harvest, the pancreas was dissected, weighed, and fixed in 4% paraformaldehyde overnight and paraffin-embedded. Tissue sections (5 μm in thickness) were immunostained with the following primary antibodies (all at 1:500 dilution): polyclonal guinea pig anti-porcine insulin (DAKO, Carpinteria, CA), mouse monoclonal anti-human glucagon (Sigma-Aldrich, St. Louis, MO), polyclonal goat anti-somatostatin (Santa Cruz, Santa Cruz, CA), and DAPI (Invitrogen, Carlsbad, CA). The primary antibodies were detected using a combination of DyLight 488, 549, and 649-conjugated secondary antibodies (1:200, Jackson Immuno Research Laboratory, West Grove, PA).

Section 2.3.6: Image Capture and Islet Quantification

As previously described [138, 139], microscopic images of pancreatic sections were taken with an Olympus IX8 DSU spinning disk confocal microscope (Melville, NY) with imaging software Stereo Investigator (SI, Micro Bright Field, Williston, VT). A modified method of ‘‘virtual slice capture’’ was used. Quantification of cellular composition (i.e., each area of β-, α-, and δ-cell populations, and islet area by automated contouring of each islet) was carried out using custom-written scripts for Fiji/ImageJ (http://rsbweb.nih.gov/ij/). MATLAB (MathWorks, Natick, MA) was used for mathematical analyses. Pancreatic endocrine cell and total islet masses were calculated by multiplying cellular or total islet area by pancreas mass.

Section 2.3.7: Histopathological Review of Pancreatic Slide Sections

5μm-thick hematoxylin and eosin (H&E)-stained sections from harvested pancreatic tissue were reviewed by a clinical pathologist on an Olympus BX41 microscope at 40X, 100X, 200X, and

400X original magnifications (OMs). All islets and ducts were examined for histologic changes, particularly lymphocytic inflammatory infiltrates. The exocrine parenchyma was examined at 40X OM entirely and was additionally reviewed to quantify mitotic figures in acinar cells in 10 high power fields (hpf) at 400X OM.

Section 2.3.7: Metabolic Cage Housing and Energy Expenditure Measurements

After 8 weeks of exposure, indirect calorimetric measurements were carried out using the LabMaster System (TSE Systems, Chesterfield, MO) on individually-housed mice and maintained under otherwise standard housing conditions (12-hour light-dark cycle; 22.2 ± 1.1°C). Mice were provided *ad libitum* access to food and water. After a 2-day acclimation period, O_2 consumption, CO_2 production, energy expenditure, locomotor activity (X-Y-axis movement activity and Z-axis rearing activity), as well as food and water consumption were monitored over 30 minute periods for 5-6 consecutive light-dark cycles over 3 successive days. The respiratory exchange ratio (RER) was calculated as the ratio of O_2 consumption to CO_2 production over 30-minute periods. These values were then averaged for each mouse during each 12-hour cycle.

Section 2.3.8: Statistical Analyses

For glucose- and insulin tolerance tests, area-under-the-curve (AUC) of blood glucose over time was calculated using the trapezoid rule-derived AUC calculation in GraphPad Prism, version 6.0. Unless otherwise noted, data are presented as means ± SEM. Statistical significance was tested using a two-tailed, Student's *t*-test to compare control and arsenic-exposed groups. Statistical

analyses were performed using GraphPad Prism, version 6.0, unless otherwise noted. $P<0.05$ was considered statistically significant for all experiments.

Section 2.4: Results

Section 2.4.1: Chronic ingestion of arsenic does not alter weight gain or total food consumption but reduces water intake

To characterize *the in vivo* effects of chronic arsenic exposure on energy metabolism, a modified paradigm from previously published studies was employed [122]. Beginning at 8 weeks of age, adult male C57BL/6J mice were fed a standard chow diet and provided *ad libitum* access to bottled drinking water or the same water supplemented with 50 mg/L sodium arsenite. Body weight, food consumption, and water intake were tracked weekly throughout the course of the study. No significant differences in body weight were found between the control and arsenic-exposed groups throughout the study, nor were there differences in final weight at the time of sacrifice (**Figure 2.4.1a**). Additionally, there was no difference in total food consumption (**Figure 2.4.1b**) (P=0.79). Water consumption was significantly lower for the arsenic-exposed animals throughout the study (**Figure 2.4.1c**), with animals in the arsenic exposed group consuming 37% less water on average. This result is consistent with results from previous rodent models using similar sodium arsenite concentrations in drinking water [136]. No outward signs of dehydration in the mice were observed, and the water intake for arsenic-exposed mice remained within the normal range of daily water intake for rodents of this strain [140].

Section 2.4.2: Arsenic exposure impairs glucose tolerance but does not impact whole-body insulin sensitivity

To determine the extent to which arsenic exposure altered glucose homeostasis, IP-GTTs were performed after 4 and 8 weeks of exposure. While glucose tolerance was unchanged at 4 weeks (data not shown), arsenic-exposed mice exhibited clear glucose intolerance by week 8 (**Figure 2.4.2a**), with a 20% increase in glucose AUC in arsenic-exposed animals (**Figure 2.4.2b**). To determine whether the observed glucose intolerance may stem from altered insulin sensitivity, IP-ITTs were performed on mice following 8 weeks arsenite exposure. Arsenic did not significantly alter global insulin sensitivity (**Figure 2.4.2c, 2.4.2d**) (P=0.53), suggesting that systemic insulin resistance was not the primary mechanism of arsenic-induced glucose intolerance in this model.

Section 2.4.3: Arsenic dysregulates steady-state glucose homeostasis

In order to determine the effect of arsenic exposure on steady-state glucose homeostasis, measures of fasting glucose homeostasis were calculated. After a 6-hour fast, arsenic-exposed mice showed trends of increased fasting glucose (**Figure 2.4.3a**) with near-significant decreased fasting plasma insulin levels (**Figure 2.4.3b**), leading to a statistically significant 28% decrease in HOMA-IR (**Figure 2.4.3c**). Although a putative index of insulin resistance, evidence suggests that the HOMA-IR falls in states of β-cell decompensation [141]. With evidence that arsenic exposure does not alter system insulin sensitivity (**Figure 2.4.3c, 2.4.3d**), the reduction in HOMA-IR suggests that the primary defect leading to arsenic-induced glucose intolerance is likely related to impaired insulin secretion from pancreatic β-cells.

Section 2.4.4: Arsenic exposure impairs insulin release in response to a glucose challenge

To assess whether arsenic impairs glucose-induced insulin release, insulin levels were quantified during the IP-GTT. Relative to the rise in blood glucose, insulin release over the first 10 minutes of the IP-GTT was reduced in the arsenic-exposed group by 19% (**Figure 2.4.4a**). This suggests a specific impairment in first-phase insulin release under conditions of arsenic exposure, as plasma insulin measured during the course of the IP-GTT was unchanged at later time points (data not shown). Collectively, these data suggest that defective insulin secretion drives arsenic-induced glucose tolerance rather than peripheral insulin resistance.

Section 2.4.5: Arsenic does not alter relative pancreatic islet endocrine cell area

Interestingly, the pancreas weight of arsenic-exposed mice was significantly lower than that of control mice (**Figure 2.4.5a**). To determine possible direct adverse effects of arsenic on the endocrine pancreas, the pancreatic endocrine cell/islet area were quantitatively analyzed in the whole pancreas. No differences were observed in β-cell, α-cell, δ-cell, or total islet area or mass between the groups (**Figure 2.4.5b, 2.4.5c, 2.4.5d, 2.4.5e**). Furthermore, there were no differences in islet cell composition, islet size distribution, or cellular composition (**Figure 2.4.5f, 2.4.5g**), suggesting that the effect of arsenic exposure on glucose intolerance is not the result of a physical loss of β-cells/islets.

Section 2.4.6: Arsenic does not have a significant effect on pancreatic islet inflammation

In order to discern whether or not inflammation or immune cell infiltration may be driving the observed insulin secretion phenotype, a pathologist was enlisted in order score histological sections for signs of inflammation and cell division, both in the endocrine islets and

in the exocrine acinar cells. Inflammatory infiltration was scored systematically in all visible islets for each histological section. No infiltration of immune cells was appreciated in any of the islets reviewed for any section in either exposure group **(Figure 2.4.6a)**. Similarly, a systematic review of inflammatory infiltrative cells in the exocrine pancreas revealed no significant differences in inflammation between groups (**Figure 2.4.6b)**, suggesting that infiltrative processes are not a major driver of the observed insulin secretory phenotype.

Section 2.4.7: Arsenic exposure alters visceral adiposity in a depot-specific manner

It is known that adipose tissue mass, and specifically the distribution of adipose tissue across different anatomical depots (e.g. visceral versus subcutaneous), has profound effects on whole-body energy metabolism, glucose handling, and insulin sensitivity [63]. To measure visceral adiposity, the three major visceral adipose depots (epididymal (or perigonadal), perirenal, and mesenteric) were completely excised and weighed upon sacrifice. After normalization for body weight, a trend towards decreased total visceral adiposity in arsenic-exposed mice was observed compared to controls (**Figure 2.4.7a**) (P=0.13). In particular, relative perirenal adipose tissue mass decreased 31% in arsenic-exposed mice (**Figure 2.4.7b**) (P<0.05). The perigonadal (**Figure 2.4.7c**) (P=0.16) and mesenteric (**Figure 2.4.7d**) (P=0.47) adipose tissue masses did not differ significantly between the groups.

Section 2.4.8: Arsenic exposure alters behavioral and metabolic circadian rhythms

In order to assess global metabolic function and behavioral parameters in live animals, mice were individually housed in metabolic cages following 8 weeks of exposure to arsenic in their drinking water. Mice were housed for a total of 5 consecutive days to allow for acclimation

and normalization of metabolic measurements, and arsenic treatment was continued throughout the metabolic caging period. Arsenic exposure markedly increased the fraction of food consumed during the normal feeding dark cycle without altering total food intake (**Figure 2.4.8a, 2.4.8b**). Indirect calorimetry measurements showed differences in energy utilization between the two groups. Specifically, arsenic exposure significantly increased the respiratory exchange ratio (RER) during the dark cycle (**Figure 2.4.8c**). Notably, no differences in locomotor activity (X-Y-axis movement) were observed between the two groups (data not shown). Furthermore, arsenic exposure increased the difference in RER between the light and dark periods (**Figure 2.4.8d**). These results demonstrate that arsenic disrupts the circadian rhythmicity of key metabolic parameters in exposed mice.

Section 2.5: Discussion and Data Interpretation

Arsenic exposure remains an important threat to human health and an underappreciated contributor to the current epidemic of diabetes. This mouse model of chronic arsenic exposure provided additional insights into the links between arsenic exposure and metabolic dysfunction. Despite normal patterns of total food intake and weight gain, arsenic exposure impaired glucose tolerance following 8 weeks of exposure without effects on systemic insulin sensitivity. Metabolic cage analyses further support the hypothesis that arsenic does not promote hyperglycemia via the induction of insulin resistance, as arsenic-exposed mice exhibited an increase in RER rather than the typical decrease observed in insulin resistant states [142]. Rather, insulin secretion was decreased during glucose challenge, suggesting that the primary cause of arsenic-induced hyperglycemia is due to an insulin secretion defect. Importantly, these disruptions do not appear to arise from a diminution of β-cell mass or alteration in islet

morphology but likely arise from defects in β-cell function. Furthermore, these studies revealed novel alterations in circadian rhythms of energy metabolism induced by drinking water arsenic exposure, thereby expanding upon previously published work.

In the present studies, the metabolic impact of arsenic was studied using exposure to sodium arsenite (As^{3+}) via drinking water. The exposure paradigm used herein was modeled on previously published work that demonstrated that mice exposed to 50 mg/L arsenite had internal levels comparable to those in highly-exposed human populations [119, 120], and utilized the most relevant species of arsenic for human exposure [143]. Importantly, rodents are generally more resistant to the toxic effects of arsenic [120]; therefore, higher concentrations are required to study its biological effects. Consistent with this prior study, mice exposed to arsenic-supplemented water in the present study consumed significantly less water than controls [136], effectively lowering the exposure relative to drinking water measurements. While mice did not display outward signs of dehydration, and previous studies revealed a decrease in hematocrit at even higher level of exposure [144], whether decreased water intake *per se* impacts the observed metabolic phenotype remains to be determined.

The data presented support the conclusion that the mechanisms of arsenic-induced glucose intolerance differ from those underlying typical T2DM. In the present study, steady-state measures of insulin resistance in the fasting state (i.e. HOMA-IR) indicated that arsenic does not promote insulin resistance. The observed reduction in HOMA-IR is in agreement with epidemiological evidence showing that greater arsenic exposure in human populations negatively correlates with HOMA-IR [108]. Rather than a typical T2DM model in which glycemic control deteriorates due to an initial loss of insulin sensitivity, arsenic likely induces glucose intolerance via a deficiency in insulin release [63]. Indeed, impairments in β-cell insulin secretion were

confirmed during IP-GTTs during which early, first-phase insulin release was reduced relative to the rise in blood glucose. Additionally, evidence that arsenic exposure tends to reduce rather than increase adiposity, an effect in agreement with prior studies [136], argues against a model of obesity-driven metabolic deterioration. Rather arsenic appears to promote a metabolically dysfunctional state similar to early type 1 diabetes (T1DM) or monogenic forms of diabetes that are characterized by primary impairments in β-cells.

In the present study, impaired insulin release and subsequent glucose intolerance were not due to overt changes in the relative area or composition of islet endocrine cells (β-cells, α-cells, or δ-cells). These data suggest that arsenic induces glucose intolerance through a disruption in β-cell function that alters normal stimulus-secretion coupling rather than a depletion of β-cell mass. In support of this hypothesis, a prior study using a cellular model of exposure demonstrated that arsenic impaired glucose-stimulated insulin secretion in a dose- and time-dependent manner [114]. Similarly, a study using isolated murine pancreatic islets found that exposure to sub-toxic concentrations of trivalent arsenic species inhibited glucose-stimulated insulin secretion [145]. Importantly, while β-cell mass was not altered by arsenic exposure in our model, total pancreatic weight was reduced by approximately 16%. Since endocrine cell mass was unchanged, this suggests that arsenic exposure reduces exocrine pancreatic mass. Interestingly, in models of T1DM in which diabetes arises from selective destruction of β-cells, pancreatic mass has similarly been shown to be reduced, possibly secondary to the loss of the trophic effects of insulin on the acinar pancreas [146]. While this does not exclude the possibility that arsenic is directly toxic to the exocrine pancreas, the current data is consistent with an induction of β-cell physiological dysfunction as the primary defect in arsenic-induced metabolic dysfunction.

Classically, arsenic has been characterized as a metabolic toxin based on its ability to inhibit intermediary metabolism at different points based on the species under investigation [135]; this includes classical evidence demonstrating inhibition of pyruvate dehydrogenase (PDH) [103]. In the current model, indirect calorimetry data demonstrated an increase in RER during the dark (feeding) phase, and an accentuation of metabolic flexibility across fed-fasted transitions. These data suggest the preferential utilization of carbohydrates for fuel during the fed state, an outcome likely to impact global metabolic parameters given prior studies in rodent models of diabetes [147]. This provides indirect evidence that arsenic exposure in the present model does not globally impair PDH, which is predicted to reduce the efficiency of carbohydrate metabolism [148]; however, tissue-specific impairments in PDH are possible. Indeed, the current data are in agreement with studies showing that β-cell-specific ablation of PDH results in glucose intolerance with concomitant hypoinsulinemia and reduced glucose-stimulated insulin secretion [149]. This raises the possibility that arsenic may have tissue-specific effects on PDH function that are not observed globally, potentially due to selective enrichment of arsenic in β-cells. Further work is required to understand the precise mode by which arsenic augments carbohydrate utilization in the fed state.

While the present studies provide critical validation of earlier models of arsenic-induced glucose intolerance and shed new light on the metabolic defects that give rise to this disruption in energy homeostasis, there remain several limitations. Importantly, dose-dependence of arsenic exposure was not assessed, and the impact of other arsenic species was not determined. Given that human populations are exposed to a multitude of arsenic species, future studies should utilize environmentally-relevant mixtures of arsenicals [97]. The current studies were also limited to male mice. Additional studies in female mice will be critical to better understand the

impact of arsenic on diabetes risk in human populations given evidence that estrogen may modulate arsenic toxicity [150].

Despite these limitations, the findings presented herein provide critical additional support to the hypothesis that chronic arsenic ingestion augments diabetes risk. Millions of people worldwide currently consume arsenic-contaminated drinking water that exceeds WHO safety limits, and arsenic contamination of groundwater remains prevalent in the United States [151]. Moreover, given recent evidence of additional arsenic ingestion from grains such as rice [152], and in populations such as East Chicago, Indiana [153], the metabolic risk imposed by arsenic is likely underestimated. To better appreciate this risk, future studies should explore potential hepatic and lipid-metabolic effects of arsenic exposure, potential synergy with other physiological stressors (e.g. caloric excess), and the molecular pathways by which arsenic disrupts β-cell stimulus-secretion coupling. Such insights will be critical for designing strategies to mitigate the deleterious effects of this ubiquitous pollutant.

CHAPTER 3

THE INTERACTION BETWEEN HIGH-FAT DIET FEEDING AND ARSENIC EXPOSURE: EFFECTS ON NORMAL METABOLIC FUNCTION IN MICE

Section 3.1: Background and Introduction

The ever-growing metabolic disease epidemic continues to impose massive economic and societal burdens on both individuals and health care systems around the globe. Dietary patterns have shifted dramatically in recent decades, and there is overwhelming evidence to support the hypothesis that consumption of "Western" diets (i.e. calorically dense diets high in simple carbohydrates and fats) contributes significantly to the development of chronic metabolic and cardiovascular diseases such as type 2 diabetes and atherosclerosis. Energy intake through the diet determines overall macronutrient availability and alters the response of individual systems and metabolic tissues to their substrates. Studies examining the relationship between diet and incidence of diabetes on an individual level have focused primarily on macronutrient intake. However, macronutrient levels alone probably explain only part of the vast array of effects that dietary changes can have on the deterioration of normal glucose metabolism.

Studies have noted significant associations between dietary patterns and plasma biomarkers associated with obesity and cardiovascular disease [154, 155]. The authors of one study determined that a Western-type dietary pattern was significantly and positively correlated with HDL cholesterol, fasting insulin and C-peptide, leptin, and C-reactive protein amongst other markers, while a so-called "prudent pattern" was inversely associated with fasting insulin levels [156]. Some studies have found that dietary fat intake is associated with clinical precursors to diabetes, including hyperinsulinemia and hyperglycemia, and even frank type 2 diabetes itself.

Perhaps not surprisingly, these results varied across different studies, and large-scale studies have failed to find consistency [157].

The combinatorial effects of dietary stressors and environmental pollutants or endocrine disrupting chemicals (EDCs) have been previously examined by several groups attempting to elucidate the contributions that these distinct and important environmental factors play in the pathogenesis of metabolic disease states in humans. In controlled experimental settings, many studies have utilized models of diet-induced obesity and diabetes in rodents to discern specific pathophysiological changes that accompany increased fat and calorie intake. Previous studies have also demonstrated additive effects of these combinatorial insults on measures of disease progression in animal models. For example, in a rat model of combined high fat diet (HFD) and bisphenol A (BPA) exposure, animals exposed to the combined insults exhibited further impairment in glucose homeostasis as compared to unexposed HFD-fed rats, although these results were not consistent across many of the other metabolic parameters tested [158]. Given that human exposure to environmental chemical pollutants occurs in conjunction with other environmental stressors, including the consumption of metabolically stressful high-fat and high-carbohydrate diets, it is important to test these combinatorial exposures in animal models in order to discern the contribution that each component may ultimately be playing in disease outcomes.

In the present study, adult male mice were grouped into a 2 x 2 experimental design where they were either challenged with a high-fat diet, or fed a purified, matched control diet, and were further divided by exposure to arsenic-supplemented drinking water versus control water. The goal of these studies was to assess the extent to which experimental manipulation of diet and macronutrient intake alters the severity of glucose homeostasis and other key metabolic

phenotypes induced by arsenic exposure. Our results indicate that while high-fat diet feeding and arsenic exposure do not promote metabolic deterioration in a synergistic or additive fashion, this combined challenge causes unique metabolic defects when compared to high fat diet or arsenic exposure in isolation.

Section 3.2: Materials and Methods

Section 3.2.1: Animal Care and Arsenic Exposure

Seven-week-old male C57BL6/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in groups of 2-3 mice per cage under 12-hour light/dark cycles at 22.2 ± 1.1°C. The number of cages with either 2 or 3 animals was equally distributed across all experimental groups. All animals received *ad libitum* access to diets as described below. The arsenic exposed groups were provided reverse-osmosis-purified bottled drinking water supplemented with 50 mg/L sodium arsenite (As^{3+}) (Sigma Aldrich, St. Louis, MO). The control water groups received the same water without arsenite supplementation. Water intake was measured weekly throughout the studies. Animals were treated humanely in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Section 3.2.2: Dietary Information

Beginning simultaneously with the onset of arsenic exposure (8 weeks of age) mice were switched onto either purified control diet (CD, Envigo Teklad Custom Diet #TD.97184) or matched high-fat diet (60% fat by total kilocalories (kcal)) (HFD, Envigo Teklad Custom Diet #TD.160079), provided *ad libitum*. Comprehensive dietary information (macronutrient and micronutrient content) can be found in **Tables 3.2.2a** and **3.2.2b**. Body weight and food intake

were measured weekly throughout the course of the study until the time of sacrifice at 24 weeks of age (16 weeks total exposure period).

Section 3.2.3: Tissue Harvest and Storage

At 16 weeks following the onset of arsenic exposure, animals were fasted for 5-6 hours and then euthanized using isoflurane anesthesia followed by exsanguination via cardiac puncture. Blood was collected in microfuge tubes, allowed to clot at room temperature for 30 minutes, and then centrifuged at 1500 x *g* for 15 minutes in order to collect serum. Adipose tissue depots (perigondal, perirenal, mesenteric, subcutaneous and intrascapular brown adipose) were dissected, weighed, and flash frozen in liquid nitrogen. Liver was collected, dissected and flask frozen immediately. Tissue samples were stored at -80°C until the time of processing.

Section 3.2.4: Intraperitoneal Glucose Tolerance Tests (IP-GTTs)

Mice were fasted for 6 hours, at which point baseline fasting blood glucose readings were obtained from all mice via tail vein sampling after application of local anesthetic (2% viscous lidocaine, Water-Jel, Carlstadt, NJ). Dextrose was injected intra-peritoneally (i.p.) at a concentration of 2 g/kg body weight. Blood glucose levels were measured at 10, 20, 30, 40, 60, 90, and 120 minutes following injection using a Freestyle Lite glucometer (Abbott Laboratories, Abbott Park, IL). Blood samples were collected from the tail vein into heparinized microtainer tubes (Sarstedt, Numbrecht, Germany) at 0, 10, 30, and 60 minutes for determination of insulin concentrations. Tubes were placed immediately on ice, centrifuged at 1500 x *g* for 15 minutes at 4°C, and plasma was collected. Plasma insulin concentrations were measured using the Mouse Ultrasensitive Insulin ELISA kit according to the manufacturer's instructions (ALPCO, Salem,

NH). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using fasting blood glucose and fasting plasma insulin levels as previously described [137].

Section 3.2.5: Intraperitoneal Insulin Tolerance Tests (IP-ITTs)

Mice were fasted for 3 hours, and fasting blood glucose levels were measured via tail vein sampling after application of local anesthetic. Mice were then injected intraperitoneally (i.p.) with Humalog insulin (0.5 U/kg body weight; Eli Lilly, Indianapolis, IN). Serial blood glucose readings were taken at 15, 30, 45, 60, 90, and 120 minutes following injection.

Section 3.2.6: Pancreatic Histology and Immunohistochemistry (IHC)

At the time of terminal harvest, pancreata were dissected from the surrounding fat and fixed in 4% paraformaldehyde overnight, transferred to 70% EtOH for storage, and then paraffin-embedded. Tissue sections (5 μm in thickness) were immunostained with the following primary antibodies (all at 1:500 dilution): polyclonal guinea pig anti-porcine insulin (DAKO, Carpinteria, CA), mouse monoclonal anti-human glucagon (Sigma-Aldrich, St. Louis, MO), polyclonal goat anti-somatostatin (Santa Cruz, Santa Cruz, CA), and DAPI (Invitrogen, Carlsbad, CA). The primary antibodies were detected using a combination of DyLight 488, 549, and 649-conjugated secondary antibodies (1:200, Jackson Immuno Research Laboratory, West Grove, PA).

Section 3.2.7: Islet Image Capture and Islet Cell Quantification

As previously described [138, 139], microscopic images of pancreatic sections were taken with an Olympus IX8 DSU spinning disk confocal microscope (Melville, NY) with the imaging software Stereo Investigator (SI, Micro Bright Field, Williston, VT). A modified method of

‘‘virtual slice capture’’ was used. Quantification of cellular composition (i.e., each area of β-, α-, and δ-cell populations, and islet area by automated contouring of each islet) was carried out using custom-written scripts for Fiji/ImageJ (http://rsbweb.nih.gov/ij/). MATLAB (MathWorks, Natick, MA) was used for mathematical analyses. Pancreatic endocrine cell and total islet areas were calculated as the average area of each fluorescent marker for each slide section (one slide section per individual mouse pancreas sample).

Section 3.2.8: Metabolic Cage Housing and Indirect Calorimetry Measurements

After 8 weeks of exposure, indirect calorimetric measurements were carried out using the LabMaster System (TSE Systems, Chesterfield, MO) on individually-housed mice and maintained under otherwise standard housing conditions (12-hour light-dark cycle; 22.2 ± 1.1°C). Mice were provided *ad libitum* access to their respective experimental diets and waters. After a 2-day acclimation period, O_2 consumption, CO_2 production, energy expenditure, locomotor activity (X-Y-axis movement activity and Z-axis rearing activity), as well as food and water consumption were monitored over 30 minute periods for consecutive light-dark cycles over 3 successive days. The respiratory exchange ratio (RER) was calculated as the ratio of O_2 consumption to CO_2 production over 20-minute periods. These values were then averaged for each mouse during each 12-hour cycle (light or dark).

Section 3.2.9: Measurement of whole-body composition by dual energy x-ray absorptiometry (DEXA) scanning

Body composition was assessed at 9 weeks following the onset of exposure with the assistance of the Metabolic Testing Facility of the Diabetes Research and Training Center (DRTC) at the

University of Chicago Medical Center. Mice were anesthetized before imaging by injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) into the intrascapular region. Body composition was measured by dual energy x-ray absorptiometry (DEXA) scanning (Lunar PIXImus densitometer system; GE Healthcare) using the PIXImus 2 software package following system calibration according to the manufacturer's instructions. Total body weight and body length were also assessed during sedation prior to DEXA scanning.

Section 3.2.10: Analysis of serum parameters

At the time of sacrifice, whole blood was obtained via cardiac puncture. Blood was allowed to clot at room temperature for 30 min, followed by centrifugation at 1,500 x *g* for 15 minutes at 4^0C. Serum parameters were quantified using commercially available ELISA kits for mouse adiponectin (Millipore, Billerica, MA) as well as colormetric assay kits for triglycerides (Cayman Chemical, Ann Arbor, MI) according to the manufacturers' instructions.

Section 3.2.11: Statistical Analyses

For glucose- and insulin tolerance tests, area-under-the-curve (AUC) of blood glucose over time was calculated using the trapezoid rule-derived AUC calculation in GraphPad Prism, version 7.0. Unless otherwise noted, data are presented as means ± SEM. Statistical significance was tested using two-tailed, Student's *t*-test for comparison of groups within each diet setting (CD or HFD), or ordinary one-way ANOVA for comparisons across all four experimental groups. Statistical analyses were performed using GraphPad Prism, version 7.0, unless otherwise noted. $P<0.05$ was considered statistically significant for all experiments.

Section 3.3: Results

Section 3.3.1: Arsenic exposure results in significantly lower weight gain in high-fat diet (HFD)-fed mice, despite no difference in caloric intake

In order to model chronic exposure to arsenic through drinking water, adult male C57BL/6J mice were provided *ad libitum* access to drinking water, either with or without arsenic supplementation, as previously described starting at 8 weeks of age. Simultaneously, mice were divided further into groups (four total experimental groups) and placed on either a purified ingredient control diet (henceforth referred to as "CD") or a matched high-fat diet (henceforth referred to as "HFD"). Data on caloric density, macronutrient and micronutrient content of the matched diets can be found in **Tables 3.2.2a** and **3.2.2b**. Based on change in total body mass from the onset of the study, mice in the HFD-fed group gained significantly less weight when exposed to arsenic, although this same phenomenon was not observed in the CD-fed mice **(Figure 3.3.1a, 3.3.1b)**. This effect was observed despite the observation that arsenic exposure resulted in no significant difference in food intake across the course of the study in either dietary group **(Figure 3.3.1c)**. Based on the caloric density of the diets (see Table 3.1), both HFD-fed groups consumed more average daily calories compared to their counterparts in the CD groups **(Figure 3.3.1d)**. As noted previously, both by our own group and by others [56], mice consuming arsenic-supplemented drinking water, in both the CD and HFD groups, drank significantly less water than controls **(Figure 3.3.1e)**. This difference was less pronounced in the HFD groups, which may be attributed to differences in palatability and macronutrient makeup of the different diets.

Section 3.3.2: Arsenic exposure significantly decreases whole-body adiposity in both dietary groups

Given the differences seen in total body mass between arsenic-exposed and arsenic-naïve mice in the two dietary groups, further experiments were executed with the aim of integrating body mass composition and adiposity. Using dual energy x-ray absorptiometry (DEXA) scanning techniques, mice body mass composition was assessed. Fat mass and lean body mass, in addition to bone mineral density (BMD), were measured in anesthetized mice following 9-weeks of exposure to experimental diet and water conditions. Whole-body adiposity (measured as %, total fat mass / total body mass) was significantly decreased in arsenic-exposed mice in both CD-fed and HFD-fed cohorts **(Figure 3.3.2a)**. This difference was apparent despite the fact that body mass was also significantly decreased in arsenic-exposed animals in both diet groups **(Figure 3.3.2b)**. These data suggest that arsenic exposure specifically affects adipose tissue expansion, and this effect is amplified when mice are fed on a HFD. Importantly, arsenic exposure did not alter total body length **(Figure 3.3.2c)** or bone mineral density **(Figure 3.3.2d)** in either dietary group. These data are important because they show that arsenic does not alter overall growth in exposed animals.

Section 3.3.3: Arsenic-exposed mice do not exhibit significant differences in glucose tolerance in either the HFD or CD groups

Based on the hypothesis that chronic arsenic exposure drives deleterious alterations in glucose homeostasis in exposed mice, serial metabolic tests were performed to quantify the extent to which exposure drives impairments in glucose tolerance. Impaired glucose tolerance is a hallmark of a human condition commonly referred to as pre-diabetes. Pre-diabetic individuals

may exhibit impaired response to a glucose challenge despite not meeting strict criteria for a type 2 diabetes diagnosis based on fasting glycemic measurements [130].

In order to assess the effects of chronic arsenic exposure on glucose homeostasis in this study, mice were subjected to intraperitoneal (i.p.) glucose tolerance tests throughout the course of the study, at 4-, 8-, and 14-weeks following the onset of exposure. The objective was to achieve a dynamic readout of the manner and timeframe in which arsenic exposure leads to changes in glucose homeostasis. Arsenic-exposed mice in both cohorts (CD and HFD) did not exhibit distinct differences in glucose tolerance when compared to matched controls at any of the time points studied **(Figure 3.3.3a, 3.3.3c)**. Area-under-the-curve (AUC) calculations for change in blood glucose over time did not demonstrate statistically significant changes between arsenic-exposed and arsenic-naïve mice in either dietary group **(Figure 3.3.3b, 3.3.3d)**. Despite this fact, as expected based on prior studies, HFD-fed mice in both exposure groups were significantly more glucose intolerant than their counterparts in the CD-fed groups, even after just 4 weeks of diet feeding.

Section 3.3.4: HFD feeding induces marked fasting hyperinsulinemia that is completely blunted by arsenic exposure

In order to more clearly elucidate the potential pathogenic role that arsenic plays in the development of diabetic and related pre-diabetic conditions, experiments were performed in order to assess circulating plasma insulin levels both under fasting and glucose-challenged conditions. When examining fasting blood glucose recorded prior to the start of IP-GTTs following a 6-hour fast, arsenic-exposed mice did not have significantly different fasting blood glucose (FBG) measures in either dietary group (CD or HFD) (**Figure 3.3.4a)**. Interestingly,

even though the mice exhibit significantly decreased weight gain and adiposity, when compared to matched HFD-fed controls, arsenic-exposed HFD mice exhibited comparable fasting hyperglycemia following 8 weeks of exposure.

However, despite no observed difference in fasting blood glucose levels, fasting plasma insulin was significantly decreased in HFD-fed, arsenic-exposed mice when compared to matched HFD-fed controls (**Figure 3.3.4b)**. In CD-fed groups, no difference in fasting insulin levels was observed between arsenic-exposed and arsenic-naïve mice.

Because of the substantial effect that arsenic-exposure has on fasting insulin levels in HFD-fed groups, HOMA-IR was significantly decreased in arsenic-exposed HFD mice when compared to diet-matched controls (**Figure 3.3.4c)**.

Section 3.3.5: Arsenic-exposed, HFD-fed mice exhibit impaired insulin secretion dynamics despite exhibiting no difference in peripheral insulin sensitivity

Following data that suggested differences in peripheral insulin resistance based on static measures, experiments were performed in order to more accurately assess insulin secretion dynamics and peripheral insulin sensitivity. Insulin responsiveness was assessed by insulin tolerance tests (IP-ITTs) in order to measure the dynamics of glucose clearance from the circulation into the periphery. CD-fed mice did not exhibit any differences in peripheral insulin sensitivity, as measured by the percent decrease in blood glucose following insulin injection between drinking water groups (**Figure 3.3.5a, 3.3.5b)**. Similarly no significant differences were present between arsenic-exposed and arsenic-naïve mice in the HFD-fed cohorts based on IP-ITT data (**Figure 3.3.5c, 3.3.5d)**.

In order to determine if the glucose intolerance observed by IP-GTT in HFD-fed mice was driven primarily by differences in circulating insulin, a time-course of plasma insulin measurements following glucose challenge during the IP-GTT was evaluated. Despite exhibiting comparable increases in blood glucose during the course of the test, arsenic-exposed, HFD-fed mice exhibited significantly decreased plasma insulin levels throughout the first 60 minutes of the test (**Figure 3.3.5e)**.

Section 3.3.6: Differences in pancreatic endocrine cell mass do not fully explain differences in insulin secretion resulting from arsenic exposure

In order to better assess the underlying pathophysiology driving the observed insulin secretory defect, pancreatic histology and islet endocrine cell composition were assessed by immunohistochemistry (IHC). The β-, α-, and δ-cell populations were quantified using antibodies targeting insulin, glucagon and somatostatin, respectively (**Figure 3.3.6)**. Total islet area was quantified as the sum total of the three individual endocrine cell markers. In order to account for the differences in size between different pancreatic tissue samples from individual animals, percent area was calculated by normalizing to total tissue area quantified. In order to normalize for pancreatic mass and body mass, relative cell mass was calculated for each mouse by multiplying cell area by pancreatic mass, and then dividing by total body mass. Within both the CD-fed groups and the HFD-fed groups, arsenic exposure resulted in no significant differences in β-cell area or relative mass, although the mean β-cell mass mirrored fasting plasma insulin levels in the groups (**Figure 3.3.6a)**. Arsenic-exposed, CD-fed mice demonstrated a significant increase in α-cell area and mass when compared to mice in all other exposure cohorts (**Figure 3.3.6b)**. δ-cell area and mass were not significantly altered by arsenic exposure in either dietary

group (**Figure 3.3.6c)**. Total islet area and mass were also not different between arsenic-exposed and arsenic-naïve mice in either dietary group, demonstrating that neither HFD challenge nor arsenic exposure, either alone or in combination, significantly alter islet cell mass over the length of a 16-week exposure time course (**Figure 3.3.6d)**.

Section 3.3.7: HFD-fed mice exhibit metabolic dysfunction compared to CD-fed mice; however, arsenic exposure does not significantly alter these metabolic parameters

In order to make behavioral assessments, parameters related to food intake, water intake and movement were tracked during metabolic cage housing. In total, food intake did not differ significantly between any of the groups, although the HFD-fed arsenic-naïve group did tend toward lower intake both during the dark cycle and overall **(Figure 3.3.7a)**. The ratio of food consumed during each light cycle, which can be used as an indicator of differences in the circadian rhythm, of energy demand, was also not significantly different between any of the groups **(Figure 3.3.7b).** As previously noted by our group and others, water intake was significantly decreased in arsenic-exposed mice as compared to naïve controls in the CD-fed groups **(Figure 3.3.7c).** Interestingly, this effect in blunted in arsenic-exposed mice in the HFD-fed group, and this observation has implications both directly for total arsenic intake and indirectly for food intake. Movement activity was measured by recording and assessing the number of x-axis movements. As expected the average number of movements was much greater for all groups during the dark cycle as opposed to the light cycle, reflecting the nocturnal nature of mice **(Figure 3.3.7d).** This data is important to track because it shows that energy utilization is not affected primarily by differences in activity, but rather by differences in energy substrate availability and energy demands of the animals.

Section 3.3.8: Circadian rhythmicity of energy substrate utilization is significantly altered by HFD consumption but not arsenic exposure

Because energy intake and utilization are essential determining factors for glucose metabolism at the organismal level, studies to assess energy intake, utilization and expenditure were performed using metabolic cages taking advantage of the method of indirect calorimetry. Mice from all four experimental groups were individually housed in metabolic cages at 8 weeks following the onset of diet and water exposure, and were allowed to acclimate for 1-2 days before the experimental measurement period began. Measurements were taken every 20 minutes throughout the day, and time course graphs were plotted in order to track values across the 12-hour light and dark cycles during each 24-hour day period.

Metabolic rate, measured as kcal expended per hour per kilogram of body weight (referred to as H(1) rate), was significantly increased during the normal active feeding period (the dark cycle) as compared to the normal resting period (the light cycle). HFD-fed mice on control water, as compared to HFD-fed mice exposed to arsenic water, exhibited a decreased metabolic rate across both the light and dark cycle **(Figure 3.3.8a, 3.3.8b)**. Based on the data showing that HFD controls have significantly greater fat mass and total body mass as compared to arsenic-exposed HFD mice **(Figure 3.3.2)**, this data suggests that arsenic-induced increases in metabolic rate may explain the reduced adiposity under high fat feeding.

In addition to total energy expenditure, relative energy utilization is another important marker of metabolic health. The respiratory exchange ratio is an estimate of the relative contribution of different primary fuel sources (carbohydrates or lipids) being used to supply an organism with substrates to meet its energy demands. RER near 0.7 are indicative of a state in which fat is the predominant fuel source, while values near 1.0 or greater are indicative of a state

in which carbohydrates are being used as the predominant fuel source. As expected, mice fed a HFD, in both arsenic-exposed and arsenic-naïve mice, exhibited significantly lower RERs when compared to their CD-fed counterparts **(Figure 3.3.8c, 3.3.8d).** This difference is significantly more pronounced during the feeding dark cycle as opposed to the resting light cycle, where RER values are generally much lower than during periods of feeding and high activity. While arsenic-exposed HFD mice trended toward an increased RER in both cycles when compared to arsenic-naïve HFD group mates, this difference was not statistically significant **(Figure 3.3.8d)**. Comparing the difference in average RER between the light and dark cycles gives a measure of so-called "metabolic flexibility", or the ability to toggle between use of carbohydrates and lipids as primary energy sources. Not surprisingly, HFD-fed mice exhibited reduced metabolic flexibility as compared to CD-fed matched counterparts in both the arsenic-exposed and arsenic-naïve groups **(Figure 3.3.8e)**.

Section 3.3.9: Adipose tissue pathology may contribute to the deterioration of metabolic health in arsenic-exposed mice

Adipokines are important hormonal factors secreted from adipocytes, and include the well-characterized members adiponectin and leptin. Studies quantifying serum levels of adiponectin and leptin in mice have often used the relationship between these two hormones in order to assess metabolic health. Using serum collected from mice following sacrifice, we quantified these important adipokines using mouse-specific enzyme-linked immunosorbent assays (ELISAs). Despite showing significantly decreased adipose mass, arsenic-exposed mice in the HFD group had significantly decreased serum adiponectin levels as compared to their more obese HFD-fed control counterparts **(Figure 3.3.9c)**. This effect was not observed in CD-

fed mice, despite the fact that arsenic-exposed mice showed a similar decrease in adiposity **(Figure 3.3.9a)**. In order to determine if adipose tissue expression of adiponectin was playing a role on the presence of circulating levels, studies to examine gene expression at the tissue level were performed. Interestingly, in both the CD-fed and HFD-fed cohorts, visceral adipose tissue arsenic-exposed mice expressed greater levels of adiponectin mRNA as compared to arsenic-naïve, diet matched controls **(Figure 3.3.9b, 3.3.9d).** Given that adipose mass is decreased in arsenic-exposed mice in both groups, the relatively increased expression of adiponectin is appears to compensate and normalize serum levels. However, in arsenic-exposed mice fed a HFD, total serum adiponectin is still significantly lower than in HFD controls.

In order to determine how differences in adiposity may be affecting other serum parameters, including serum lipid levels, serum triglyceride levels were profiled. Elevated serum triglyceride levels are one component of metabolic syndrome, and have been shown to correlate with hyperglycemia and an increased risk for developing diabetes in human patients. No significant differences in triglyceride levels were noted between arsenic-exposed and arsenic-naïve mice fed on either CD or HFD, although there was a trend ($p=0.06$ and 0.08, respectively) towards decreased TG levels in arsenic-exposed mice in both diet groups **(Figure 3.3.9c, 3.3.9e)**.

Section 3.4: Discussion and Data Interpretation

While prior publications using animal models of drinking water arsenic exposure have attempted to model the effects of high-fat diet (HFD) feeding on metabolic dysfunction, the studies described in this paper are the first to take a comprehensive approach to describing alterations in pancreatic islet and insulin dynamics as they relate to changes in glucose metabolism in mice. The results described in the present studies verify previous observations

from similar mouse models used by other groups, but more importantly, expand on those observations and provide physiological and mechanistic reasoning to describe phenotypes related to arsenic-induced alterations in glucose homeostasis.

In prior studies conducted by our group, summarized in this dissertation in Chapter 2, following chronic exposure to arsenic through drinking water, mice fed a standard, natural ingredient laboratory control diet developed significant glucose intolerance as compared to matched controls in only 8 weeks. Based on data described here, mice exposed to arsenic, both following 8 weeks and 14 weeks of total exposure and fed either CD or HFD, did not develop glucose intolerance as compared to arsenic-naïve controls. This observation underscores the substantial effects that diet and nutritional background can have on metabolic phenotypes in animal models. Prior studies by other groups using purified laboratory diets have reached similar conclusions [56]. Levels of certain key micronutrients differed substantially between the natural ingredient diet and the purified diet used for these studies (see **Table 3.2.2b**). Although the macronutient parameters of the CD in this study are on par with standard laboratory control diets (see **Table 3.2.2a**), the source of these macronutrients is vastly different on a purified vs. natural ingredient background.

It is well appreciated that dietary patterns play a profound role in driving glucose homeostasis in humans. For example, studies looking at "western dietary patterns", grouped to contain higher proportional intake of high-fat dairy products, refined grains and processed meats, found that a western dietary pattern was associated with a significantly increased risk for developing type 2 diabetes in adult males, even independent of BMI, physical activity, family history of diabetes, and age [154]. Future studies looking at interactions between diet and other environmental stressors in the context of metabolic health need to model these factors with

increased precision. Given that human populations in regions with the highest levels of drinking water arsenic exposure (e.g. Bangladesh, Cambodia) follow a dietary pattern that is distinct in both its macro- and micronutrient content as compared to Western-type diets, diets that more closely mimic the nutritional content of native diets in the developing world may reveal unique metabolic consequences when combined with arsenic exposure in animal models. Other groups have used rodent diets mimicking native human diets to discern effects on growth and metabolic health. For example, studies looking at the effects of microbiota on undernutrition and growth used a mouse model diet formulated based on a dietary survey of complementary feeding practices in Malawian infants, the target population of interest in this particular study [159]. The studies described within are an important step towards a greater understanding of arsenic-diet interactions in the context of a chronic EDC exposure.

Notable differences between the overall body weight of arsenic-exposed and arsenic-naïve mice were apparent early in these studies, particularly for the HFD cohorts. The total body mass curves between the HFD-fed groups began to diverge at approximately 2-3 weeks in the course of the study. Despite this observation, no differences in food consumption, either in manual measurements made weekly throughout the course of the study or in automatic food consumption measurements taken during individual caging in metabolic cages, were observed between arsenic exposure groups within each of the dietary groups. The differences in weight gain are likely attributable in large part to significant differences in adipose mass between arsenic-exposed and arsenic-naïve mice in the two diet groups, although other sources contributing to loss of body mass (i.e. specific organ weights) should be probed in future studies in order to determine the source of decreased mass more precisely.

Despite significant differences in weight gain and whole-body adiposity measurements over the course of the exposure period, arsenic-exposed mice did not have reduced energy intake, that is they did not consume fewer calories, than their arsenic-naïve counterparts. This may suggest numerous potential possibilities that may explain mechanisms by which arsenic exposure ultimately contributes to global metabolic dysfunction in these mouse models. Absorption of calories from the digestive track may be influenced by arsenic exposure consumed simultaneously through the drinking water. One hypothesis that may explain altered metabolism and weight gain in the context of similar energy intake is that changes in the composition of the gut microbiome may lead to or exacerbate human disease states. Past studies have shown that arsenic can alter that composition of the gut microbiota in exposed mice, and data indicated that exposure not only perturbs the composition of the microbiota at the abundance level, but more importantly alters the microbiota metabolite profile [160]. Future studies should be designed in order to provide mechanistic reasoning that can link arsenic-induced metabolite changes to alterations in nutrient absorption or barrier function, key factors that may ultimately influence host metabolism and disease.

Glucose intolerance as assessed by glucose tolerance testing, provides an adequate measure of perturbations to global glucose homeostasis in animal models. To date, multiple studies in humans have utilized glucose tolerance testing as a way to quantify diabetes risk in populations exposed to high drinking water arsenic concentrations [112]. Impaired glucose tolerance and fasting hyperglycemia are hallmarks of diabetes, and although arsenic exposure did not alter either parameter in the current studies, HFD feeding was sufficient to induce the development of a diabetic phenotype after only 8 weeks of exposure. Interestingly, the development of impaired glucose tolerance on HFD as compared to CD was independent of

obesity, given that both arsenic-exposed and arsenic-naïve mice in HFD groups displayed comparable glucose intolerance despite arsenic-exposed mice having significantly lower fat mass. In this case, it seems likely that although overall glucose tolerance as measured by IP-GTT is similar between the HFD-fed groups, the origin of this impairment is distinct for each exposure group.

Steady-state measures of insulin resistance calculated using fasting measures (i.e. HOMA-IR) suggested that arsenic exposure does not lead to outright peripheral insulin resistance. Reduced HOMA-IR in HFD-fed, arsenic-exposed mice as compared to HFD-fed controls is consistent with data from epidemiological studies showing that greater arsenic exposure in human populations is negatively correlated with HOMA-IR measures. The level of insulin secretion over time following the onset of glucose challenge (during the IP-GTT) was inadequate to control blood glucose levels in arsenic-exposed, HFD-fed mice. Greater insulin secretion and higher systemic circulating insulin levels in HFD-fed controls likely adjusts for greater peripheral insulin resistance in adipose tissue, which in the obese mice would be a major sink for glucose disposal. It is known that skeletal muscle is responsible for the vast majority of glucose disposal, through insulin-responsive glucose uptake, in the periphery. Follow-up experiments should assess insulin resistance at the tissue level, including in skeletal muscle, in order to determine the role that muscle glucose disposal plays in arsenic-induced metabolic dysfunction.

Indirect calorimetry through the use of metabolic caging is commonly utilized to discern drivers for metabolic phenotypes in rodent studies [142]. Describing energy balance and global energy utilization at the organismal level is crucial for the characterization of disruptions to normal glucose-insulin homeostasis in states of caloric excess (i.e. HFD challenge) [98]. As

such, the principles of indirect calorimetry were applied for describing outcomes in the mouse models used for these studies. Surprisingly, significant differences in observed metabolic parameters between arsenic-exposed and arsenic-naïve animals were not immediately apparent, even in HFD animals. Further studies will be necessary in order to properly interpret the data gathered here using indirect calorimetry methods. Additionally, determining cause and effect between behavioral parameters

Adipokines, hormones produced and delivered into circulation primarily from adipocytes, play an essential role in controlling metabolic health in mammals. As an example, serum adiponectin concentrations often serve as a biomarker for insulin resistance and metabolic dysfunction [161]. Interestingly, its expression and secretion are diminished in the context of increased visceral adiposity, despite the fact that overall fat mass is greater in these scenarios [162]. Adiponectin promotes metabolic health and is linked to increased fatty acid oxidation and suppressed hepatic glucose production in the liver, improved β-cell function, and enhanced insulin sensitivity in peripheral tissues [162]. Circulating leptin also originates primarily from adipocytes, and serum leptin levels are tightly correlated with adipose mass. Leptin exerts its systemic effects via specific receptors in the central nervous system (CNS) and in the periphery outside the CNS. Here we have shown that arsenic-exposure decreases circulating adiponectin levels in the context of HFD-feeding, even though mice simultaneously exhibited decreased adipose mass. This is in addition to the observation that adipose tissue from HFD-fed mice exposed to arsenic has increased adiponectin expression. These data suggest that arsenic exposure can alter adipocyte function at the tissue level, although whether or not decreased circulating adiponectin levels directly alter insulin sensitivity in specific peripheral tissues in the context of decreased obesity remains to be determined. Our data suggest that peripheral insulin

sensitivity, as a whole, is unchanged by arsenic exposure. However, it remains to be seen if the insulin sensitivity of different tissues involved in glucose uptake (i.e. muscle, liver, adipose tissue) is differentially altered.

Serum lipid parameters are well appreciated as contributing factors to cardiometabolic diseases and diabetes. Circulating levels of LDL cholesterol, HDL cholesterol, free fatty acids (FFAs), and triglycerides (TGs) are all known to positively or negatively correlate with risk for developing metabolic and cardiovascular disease [163]. Because total intake of dietary fat was equal in the HFD groups regardless of arsenic exposure status, we determined serum lipid levels in order to ascertain whether arsenic-induced adipose loss was resulting in increased circulating lipid species. No significant differences in triglyceride levels were noted between arsenic-exposed and arsenic-naïve mice fed on either CD or HFD, although there was a trend (p-values=0.06 and 0.08 respectively) towards decreased TG levels in arsenic-exposed mice in both diet groups. Given that adipose mass is decreased in arsenic-exposed mice, lower serum triglyceride levels are not unexpected. However, increased serum triglycerides have been observed in other models exhibiting decreased adipose tissue accretion (e.g. non-alcoholic fatty liver disease (NAFLD) mouse models) and would be indicative of increased lipid flux out of adipose tissue and into the circulation. Lipotoxicity associated with circulating excess lipid and ectopic lipid accumulation in other metabolic tissues (i.e. hepatic steatosis in NAFLD) can lead to inflammation and dysfunction at the cellular level. These lipotoxic states can drive alterations in normal hepatic insulin signaling and β-cell glucose responsiveness, and these processes in turn can both drive systemic impairments in glucose homeostasis [164]. Whether or not these tissue level defects are present in arsenic-exposed individuals remains to be determined, and future studies using animal models should specifically test these questions at the cell signaling level.

Although the experimental data presented here expand upon the current knowledge base concerning the potential diabetogenic and metabolically disruptive effects of chronic arsenic exposure, future studies should focus on more clearly delineating the molecular mechanisms, at the cellular and tissue levels, that are directly disrupted by arsenic species. The elucidation of these mechanisms will require both tissue-level analyses in essential metabolic organs (i.e. liver, adipose tissue, skeletal muscle), as well as *in vitro* studies in cellular models. For studies of tissues and organ systems, insulin sensitivity and glucose disposal should be assessed using hyperinsulinemic-euglycemic clamping, the gold-standard method to assess insulin sensitivity *in vivo*. The use of radioactively-labeled glucose during clamping makes the measurement of glucose metabolism in individual organs a possibility, and this data would provide a more complete picture of the various detriments driving arsenic-induced metabolic dysfunction. Additionally, in order to study the direct effects of arsenic on insulin secretory capacity, functional studies in isolated pancreatic islets must be performed. Based on our data showing that insulin secretory capacity following glucose challenge is diminished in HFD-fed, arsenic-exposed animals despite only minor reductions in pancreatic β-cell mass, it would be worthwhile to pursue more in depth testing of insulin secretion under glucose-stimulated conditions.

It is clear that the classical model of adult type 2 diabetes development (i.e. peripheral insulin resistance brought on by aging and overt obesity, eventually leading to impaired glucose regulation, and ultimately progressing to frank diabetes) does not fit cleanly with observations from these mouse models. Data from this study support the conclusion that the mechanisms of arsenic-induced metabolic dysfunction differ from those classically driving the development of type 2 diabetes in adult humans. Importantly, this may ultimately change the way that clinicians view patients with certain environmental risk factors, including heavy metal exposures.

In order to continue elucidating the role that arsenic and other harmful environmental pollutants play in the exacerbation of human disease states, basic science studies will need to continue to pursue the mechanistic underpinnings of their biological action. Because humans are exposed to countless different environmental insults throughout the course of their lifetime, potentially acting synergistically or antagonistically to one another, the importance of combinatorial exposure studies cannot be overstated. Additionally, using genetically modified mouse models would allow researchers to discern the role of essential cellular signaling pathways, specifically as they relate to the physiological response to arsenic exposure, in different metabolic tissues. Ultimately, a better understanding of how metabolic tissues respond to the insult of chronic arsenic exposure will allow clinicians to make more informed decisions when they are considering treatment options for patients who are exposed to these environmental risk factors.

CHAPTER 4

FUTURE DIRECTIONS FOR BASIC RESEARCH DIRECTED TOWARDS ENVIORNMENTAL ARSENIC EXPOSURE AND RELATED HEALTH OUTCOMES

Section 4.1: Next Steps for Arsenic Exposure Research Using Animal Models

While the research detailed in this dissertation acts to significantly advance the study of metabolic outcomes as they relate to environmental chemical exposures in animal models, there remains significant work still to be done. These studies have utilized mouse models of chronic arsenic exposure through drinking water in order to most accurately model the route of exposure most typical in humans. While from a functional perspective this paradigm appears to be the most pragmatic, there may exist other *in vivo* dosing methods that would provide exposure doses more relevant for modeling human health outcomes. In order to increase consistency across animals and experimental cohorts, dosing arsenic in bolus-type exposures on a daily basis may prove to be more relevant. Other groups have used similar exposure models in the past for a diverse set of EDC studies; however, the rate at which organisms are exposed to chemicals likely plays an important role in the metabolism and detoxification processes for these chemicals, and as such may not be as relevant for modeling chronic, low-dose human exposure conditions. Unfortunately, it has proven difficult to understand which specific exposure models most accurately mimic human exposure with the greatest accuracy. This point exemplifies the need to integrate research findings from many different models when considering their translational potential in humans.

More generally, considering the field of EDC research in its entirety, there has been a distinct lack of studies that use a combinatorial approach including genetically modified animal

models and co-exposure to environmental pollutants. Some studies have utilized genetically-modified models in order to model disease conditions (e.g. ApoE$^{-/-}$ mice for atherosclerosis research), but few if any have utilized tissue-specific knockouts models to elucidate specific mechanistic phenotypes related to harmful chemical exposures [165]. Researchers must design studies to more fully utilize genetic manipulations in order to tease out the cellular targets that play a critical role in regulating the deleterious effects seen following exposure to arsenic.

Because it is well appreciated that dose of exposure and the time course of exposure both play a large role in determining physiological outcomes, future studies must focus on defining the relative risk of disease outcomes based on different exposure levels and time courses. As discussed in Chapter 1, arsenic speciation plays a key role in determining bioavailability, and total arsenic exposure varies widely between individuals and across different communities. The more accurate laboratory models can be at recapitulating these conditions in controlled settings, the better the translational potential of the research findings will be. Additionally, it will be crucially important to address the following aspects of metabolic health and disease, outlined in Sub-Sections 4.1.1 to 4.1.5, as they relate to chronic arsenic exposure models.

Section 4.1.1: Molecular Mechanisms of Arsenic Disruption of β-cell Secretory Function

Based on the results described by the current set of experiments, the conclusion is that arsenic-exposure drives glucose intolerance and diabetic phenotypes primarily by disrupting normal insulin secretion. In order to understand the molecular mechanisms involved in this process, it will be necessary to design both *in vitro* and *in vivo* experiments that can probe arsenic-induced glucose-insulin stimulus uncoupling at the cellular and sub-cellular levels in pancreatic islets, and specifically in β-cells. The use of pancreatic β-cell cultured cell lines

(including MIN6-K8, INS-1, and NIT-1 cells amongst others) will prove useful for testing how different arsenic species (and different concentrations of these chemicals) alter insulin secretion in the presence of glucose stimulation. Past studies have used cell lines to study the effects of arsenic on glucose-stimulated insulin secretion (GSIS), but they are limited in scope and mechanistic detail [71, 114]. By understanding disruption at the level of cell signaling pathways or cellular energy metabolism, strategies to mitigate arsenic-induced islet dysfunction can identify and hit the must relevant targets. Current collaborative studies with the Seino Lab at Kobe University School of Medicine in Japan are working to understand how changes in intracellular bioenergetics pathways may drive arsenic dysfunction specific to β-cells.

Section 4.1.2: Interactions between Arsenic and Other Environmental Stressors

As is understood from the ubiquity of harmful chemical stressors in our environments, no single pathogenic factor acts alone in the real world in free-living settings. As described in this dissertation, dietary background can play a significant role in defining experimental outcomes with regards to metabolism. As has been shown in numerous studies, the nutritional state of an organism, in terms of dietary composition, can have profound effects on phenotypic outcomes of outside metabolic perturbations [158, 159]. Accurately modeling the diet of populations exposed to high levels of arsenic would prove beneficial for a number of reasons. First, there is an issue of social justice concerning populations in developing countries and their disproportionate exposure to environmental contaminants. Arsenic exposure is most strongly associated with metabolic disease incidence in certain human populations [107, 109]. Mimicking the nutrient state of these populations takes into account diet-arsenic interactions as potential key determinants of disease pathogenesis.

Additionally, studying dietary differences in areas of high arsenic-associated disease burden in the developing world may be able to provide novel insights into the nutritional factors that sensitize to arsenic-mediated metabolic disruption. Establishing a mouse model utilizing a novel diet that aims to recapitulate key macro- and micronutrient features of developing areas where exposure to arsenic-contaminated groundwater is highly problematic should be considered. Critically, dietary deficiencies in the developing world may directly modulate pathways susceptible to arsenic disruption in experimental models (e.g. iodine, vitamin A, and zinc deficiency). It is our hypothesis that these dietary modifications (i.e. diets composed primarily from carbohydrates and containing key micronutrient deficiencies) will result in metabolic perturbations that will ultimately modify the magnitude of arsenic exposure phenotypes, and therefore provide more accuracy for the determination of disease susceptibility in exposed populations. In one example of a relevant clinical study in humans, the Bangladesh Vitamin E and Selenium Trial (BEST), a population-based, double-blind, randomized controlled trial of 7,000 adults with skin lesions, was performed in order to test the effects of micronutrient supplementation for the prevention of non-melanoma skin cancer [166]. On the other side of spectrum, some nutrients may actually increase arsenic toxicity at high doses, as has been potentially noted for selenium [167]. As is evident from the lack of consensus, additional research is needed in order to better understand how the balance of various nutritional factors influences disease outcomes.

As of the writing of this dissertation, additional studies in the Sargis Lab have begun to look at ways in which arsenic-exposure phenotypes in mice are altered by changes to selenium intake and selenoprotein biology. Preliminary studies of $Sbp2^{+/-}$ mice (global heterozygous knockout mice) exposed to arsenic indicate that selenoprotein deficiency amplifies the

detrimental effect of arsenic on glucose tolerance and impairs normal patterns of insulin secretion (**Figure 4.1.2a, 4.1.2b**). More specifically, $Sbp2^{+/-}$ mice fed a selenium-deficient (0.01 ppm) diet in the presence or absence of 50 mg/L As^{3+} in their drinking water for 8 weeks developed significantly more severe glucose intolerance compared with either wild-type mice (see Chapter 2), or arsenic-naïve $Sbp2^{+/-}$ controls. Studies are ongoing in order to determine the relative contributions of impaired insulin secretion versus insulin resistance to the observed glucose intolerance phenotype. Future work will more closely dissect the mechanisms responsible for impaired glucose tolerance in arsenic-exposed $Sbp2^{+/-}$ mice. Furthermore, studies in which tissue-specific deletions of specific selenoproteins (e.g. glutathione peroxidase proteins) are created and phenotyped will be imperative for ultimately pinpointing potentially druggable targets involving selenium metabolism and arsenic metabolism *in vivo*.

Section 4.1.3: Defining the Role of the Liver in Arsenic Detoxification and Concurrent Glucose Metabolism Under Conditions of Arsenic Exposure

Arsenic is retained in high amounts by the liver, which is not surprising given that the liver plays such an important role in the arsenic methylation and detoxification process. Because the liver is functional in a dual role, also being responsible for glucose storage in the form of glycogen and the production of glucose through gluconeogenesis, future studies examining the effects of arsenic on hepatic glucose metabolism will be of particular interest. These studies can be performed using *in vitro* cell models, primary hepatocyte models, or in animal studies *in vivo* by directly examining metabolic signaling pathways in the liver through changes in expression or insulin sensitivity. As with other metabolic tissues, it will be important to determine which

changes induced by arsenic are functioning as disease drivers, and which functions are simply compensatory responses to primary effects of exposure.

Ectopic distribution of lipid in non-adipose tissue locations is a well-known contributor to many diseases, including non-alcoholic fatty liver disease (NAFLD) and its progressive form non-alcoholic steatohepatitis (NASH). The phenomenon of lipid deposition in the liver, termed hepatic steatosis, can disrupt normal glucose metabolism and related metabolic functions, including proper detoxification [168]. It will be interesting to see whether arsenic-induced metabolic outcomes are driven in part by mechanisms related to liver detoxification, since disruption of cellular energy metabolism can ultimately change cellular functionality from a detoxification standpoint.

Section 4.1.4: Defining the Role of Adipose Tissue Function in Arsenic-Driven Metabolic Disease

Because adipose tissue is now appreciated as an essential, dynamic endocrine organ, as opposed to a passive energy storage depot, it's role in environmentally mediated disease processes must be more completely defined. White adipose tissue (WAT) in and of itself plays unique and occasionally antagonistic roles depending upon its anatomical location. Subcutaneous WAT is not classically associated with increased cardiometabolic disease risk (and may even be associated with decreased risk in some settings, whereas visceral WAT accumulation is associated with metabolic disease [162]. The experiments detailed in the current work do not make a distinction as to whether the adipose mass lost following arsenic exposure is preferentially lost from subcutaneous vs. visceral depots. Because this would have significant

implications for disease risk, follow-up studies should examine the depot specific effects of adipose tissue loss following chronic arsenic exposure.

Similarly, different developmental origins may drive distinct phenotypes related to adipose tissue function. Brown adipose tissue (BAT), which originates from distinct precursor cell populations as compared to WAT, plays a central role in regulating energy expenditure through thermogenesis, and it is appreciated that so-called "browning" or "beiging" of WAT may confer metabolic health benefits [169]. The signaling pathways controlling WAT beiging (e.g. β_3-adrenergic receptor-mediated signaling) have not been examined in the context of arsenic exposure, but given the effects seen on energy expenditure in HFD-fed animals in described in Chapter 3, this avenue of experimentation may prove fruitful for uncovering the mechanistic basis of arsenic-induced alterations in energy utilization and energy expenditure.

Section 4.1.5: Potential Contributions of Changes to Gut Health in Metabolic Dysfunction Related to Arsenic Exposure

It is known that gut hormones play an important endocrine role in the control of blood glucose levels. These hormones are defined as the incretin class of gut-derived peptides, which includes glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). In terms of blood glucose control, they act to stimulate insulin release and suppress glucagon release, ultimately resulting in the increases clearance of blood glucose following a meal [63]. They also mediate food intake, gastric emptying, energy expenditure, inflammation and a number of other essential metabolic processes. Because the phenotypes observed in these studies are affected in large part due to changes in energy expenditure, despite no clear differences in food intake, it will

interesting to see whether or not changes to gut hormone release or action play a role in arsenic-induced metabolic outcomes.

The past decade has seen an explosion in the number of publications describing research aimed at uncovering the role that the human gut microbiome plays in influencing disease pathogenesis. The diseases of primary interest in these studies include everything from immune-mediated disorders to neurological conditions to metabolic disease and diabetes [159, 170]. Studies using animal models have also described consequences of arsenic exposure as they relate to pathogenic changes in the composition of the gut microbial community, commonly referred to as dysbiosis. For example, researchers from one group used an integrated approach that combined 16S rRNA gene sequencing and mass spectrometry–based metabolomics profiling in order to examine the functional impact that arsenic has on microbiota composition and subsequent fecal metabolite profiles in exposed mice [171]. While these results are intriguing on their own, further studies are necessary in order to establish potential links between arsenic-induced changes in gut microbiota composition and global metabolic outcomes, including changes in normal glucose homeostasis.

Section 4.2: Assessing and Alleviating the Impact of Metabolic Dysfunction on Health Outcomes Driven by Arsenic Exposure

Future efforts to control metabolic disease that may be exacerbated by exposure to arsenic in the environment must focus on mitigating deleterious outcomes at all stages of the exposure and follow-up process. While ongoing efforts to clean and purify water sources and educate populations in regions with heavily contaminated drinking water sources will ultimately prove to be most beneficial in eliminating negative outcomes associated with exposure, millions

of people worldwide have already been detrimentally exposed. As the disease burden from environmental chemical exposures continues to worsen, health care systems will continue to be overwhelmed by

Recently, many research efforts from top environmental scientists have focused on defining methodologies for assessing burden from diseases thought to be driven, at least in part, by environmental chemical exposures [132]. In order to address reports concerning EDC literature that criticize the field generally for failing to use transparent and objective approaches to make conclusions about the strength of evidence necessary to link EDC exposures to detrimental health and environmental outcomes, a group of researchers developed a review framework for systematic review and integrated assessment (SYRINA) of studies concerning exposure to EDCs [172]. This methodology uses an approach designed to integrate evidence coming from multiple different "upstream" sources in order to draw conclusions, make recommendations, and evaluate uncertainties. By providing a thorough, scientific approach to EDC risk evaluation, approaches such as this one help to legitimize and integrate multitudes of studies for different exposures.

Section 4.3: Potential Modifications to Clinical Practice Resulting from Better Understanding of the Environmental Contributors to Chronic Human Disease

Although numerous challenges remain, the current level of scientific evidence supports the hypothesis that deleterious health consequences can arise from our exposure to environmental toxicants. It is my sincere hope that future scientific advances, both at the population and basic science levels will permit the scientific and public health communities to better address this important issue through the development of novel targeted therapies,

improvements in environmental remediation, and the championing of sound public policy at both the local and federal levels. Ultimately the goal of performing basic research is to be able to translate the benefits into commercial products, research techniques, or practices that benefit society and human health on an international scale. Clinical practice for T2DM is ever evolving, with new treatment designs and therapeutic targets being characterized and developed constantly[173-176]. These treatments often specifically target certain metabolic tissue types, and better characterization of clinical outcomes and disease risk following exposure to EDCs, including arsenic, can and should play a role in public health decisions to follow. Decisions regarding regulatory action need to be dependent not only on the strength of evidence provided from basic research and epidemiological studies, but also on the perceived magnitude of downstream consequences stemming from action vs. inaction for individual cases.

Section 4.4: The Future of EDC Research and the Role of Basic Scientific Research in Informing Public Policy Decisions

Sound public policy needs to be written and enacted specifically to address the most pertinent environmental issues of our time. These policies need to come from a place of compassion and concern for the health of our global citizens and for future generations. Environmental protection, especially for those who are disproportionally affected by harmful chemical exposures, cannot come from a mindset driven purely by potential profitability. Decision makers are faced with difficult choices concerning when and where to act on environmental pollutants in different situations. Given the necessity for swift action before harm to human health or environments arises, especially if such effects are irreversible in nature, the potential consequences of inaction cannot be overstated. As of the writing of this dissertation, the

current political climate in the United States is one in which Congress and the Executive administration are not responsibly addressing the implementation and funding of essential programs designed to foster basic scientific research discoveries at an adequate level. Basic research needs to be funded, at the very least, to an extent where collective knowledge produced from the intellectual economy as a whole can sufficiently inform the prioritization of scientific funding decisions in the most practical and immediate manner. Even more importantly, continued funding at high levels has the potential to transform the backbone of the United States and world economies in ways that improve our collective well being beyond what is even deemed possible by current standards [177].

Section 4.5: Final Conclusions

As is the case for any research project of significance to global health outcomes, it is my sincere hope that the research described here in this dissertation plays an important role in defining and informing future research directions for the field of endocrine disrupting chemicals and environmental health. Scientists should be judged not by what they have accomplished for personal gain, but what they have done to help improve their communities and society as a whole. I hope that as I move ever forward and continue to evolve in my own scientific career that this dissertation acts as a base from which I can grow, continue to expand my knowledge, and positively increase my impact on the biomedical research community.

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Appendix A: Figures

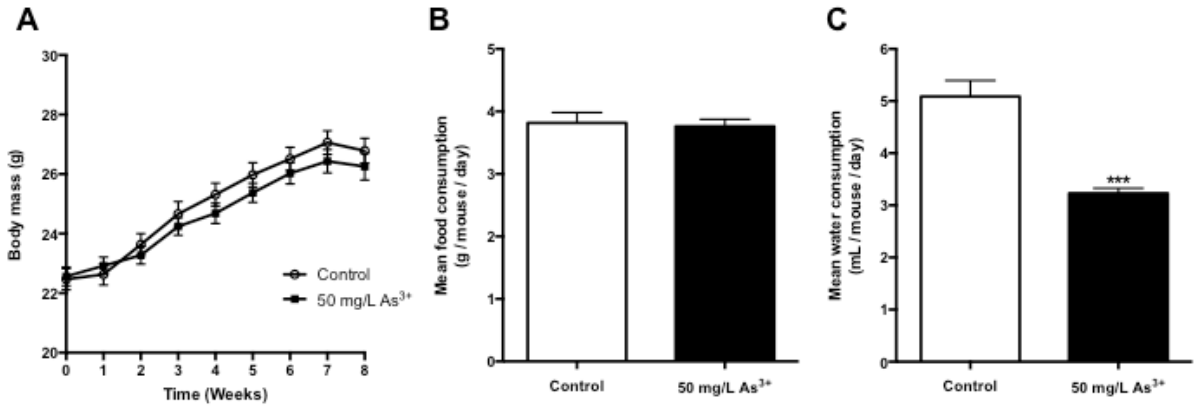

Figure 2.4.1: Chronic ingestion of arsenic does not alter weight gain or total food consumption but reduces water intake

(a) Total body mass as measured weekly over the course of the study. (b) Mean food consumption measured over the course of the study. (c) Mean water consumption measured over the course of the study. n= 16 mice per group. Figures show mean ± SEM. Statistics: Ordinary one-way ANOVA corrected for multiple comparisons (2.4.1a) or unpaired Student's t-test (2.4.1b, 2.4.1c). ***$p<0.001$.

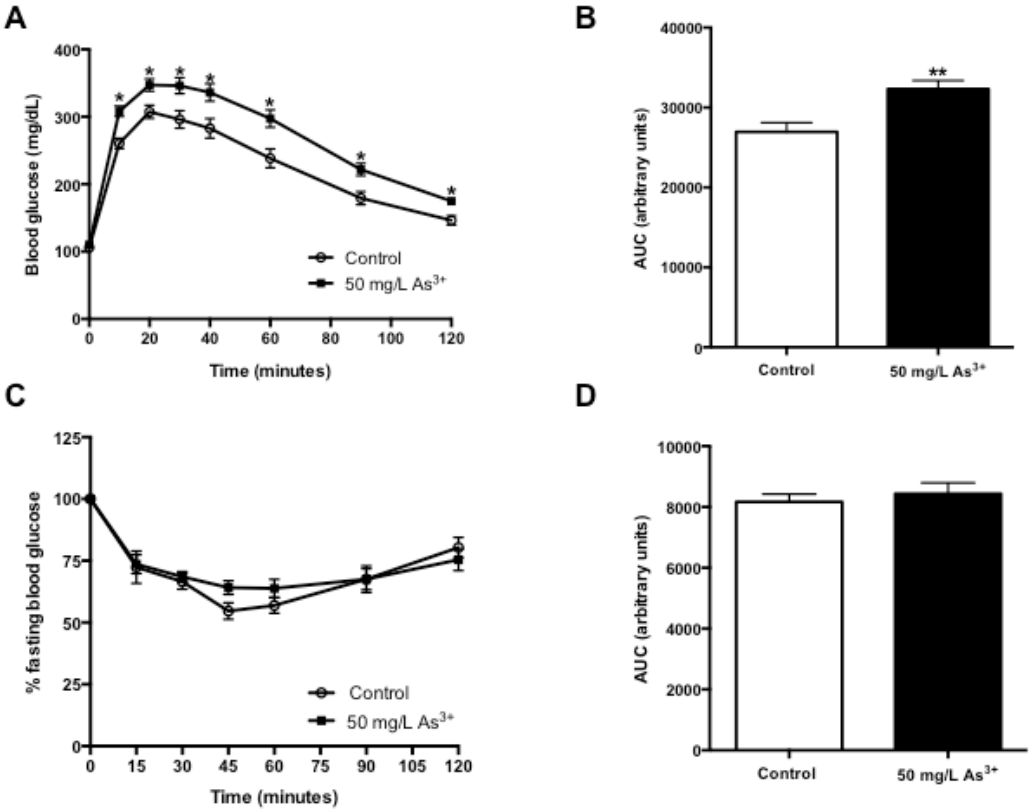

Figure 2.4.2: Arsenic exposure impairs glucose tolerance but does not impact whole-body insulin sensitivity

(a) Intraperitoneal glucose tolerance tests (IP-GTT) performed following 8 weeks of exposure. n= 24-26 mice per group. (b) Area-under-the-curve (AUC) from the IP-GTT. (c) Intraperitoneal insulin tolerance tests (IP-ITT) performed following 8 weeks of exposure. n= 9-12 mice per group. (d) AUC from the IP-ITT. Data represented as mean ± SEM. Statistics: Ordinary one-way ANOVA corrected for multiple comparisons (2.4.2a, 2.4.2c) or unpaired Student's t-test (2.4.2b, 2.4.2d). *$p<0.05$, **$p<0.01$.

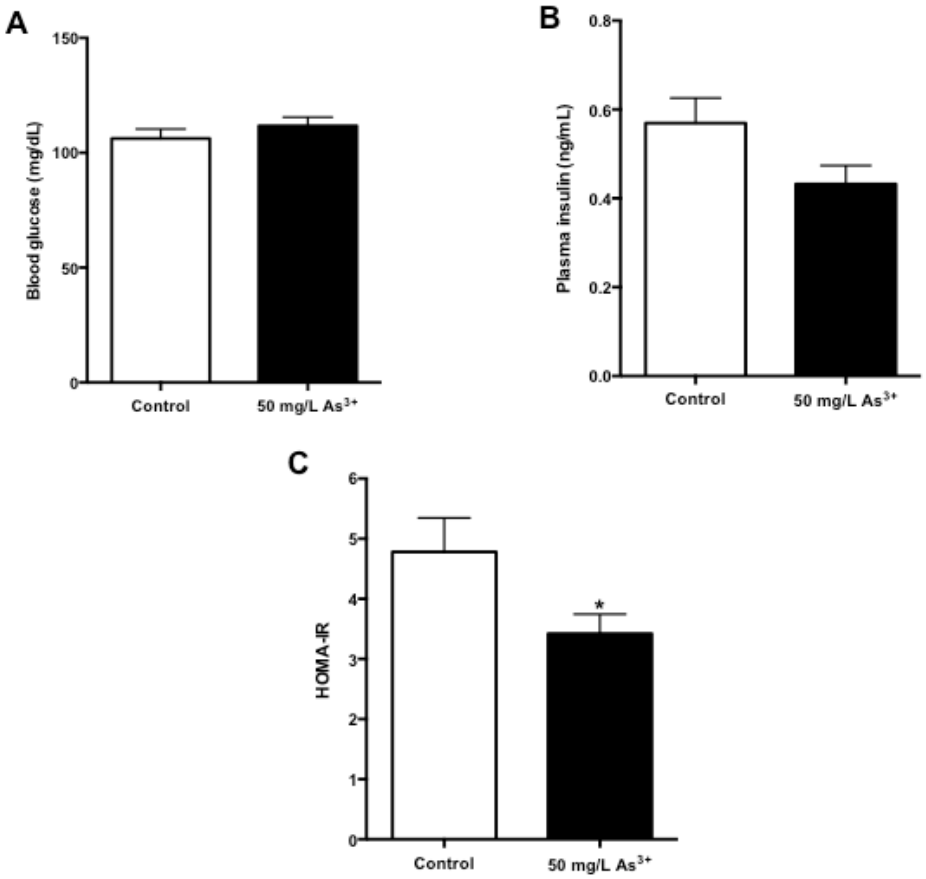

Figure 2.4.3: Arsenic dysregulates steady-state glucose homeostasis
(a) Fasting blood glucose following a 6-hour fast. (b) Fasting plasma insulin collected from peripheral blood following a 6-hour fast. (c) HOMA-IR values calculated using fasting blood glucose and fasting plasma insulin measures. n= 14-16 mice per group. Figures show mean ± SEM. Statistics: *$p<0.05$ by unpaired Student's t-test.

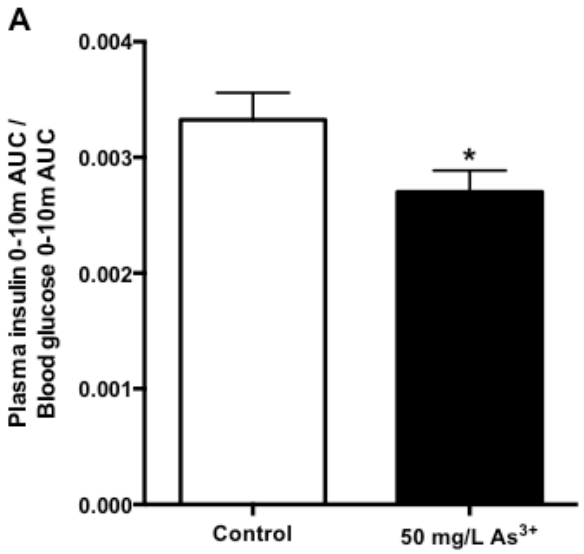

Figure 2.4.4: Arsenic exposure impairs insulin release in response to a glucose challenge
(a) Change in insulin (AUC) relative to the change in blood glucose (AUC) between 0 to 10 min during an IP-GTT. n= 14-16 mice per group. Figures show mean ± SEM. Statistics: *$p<0.05$ by unpaired Student's t-test.

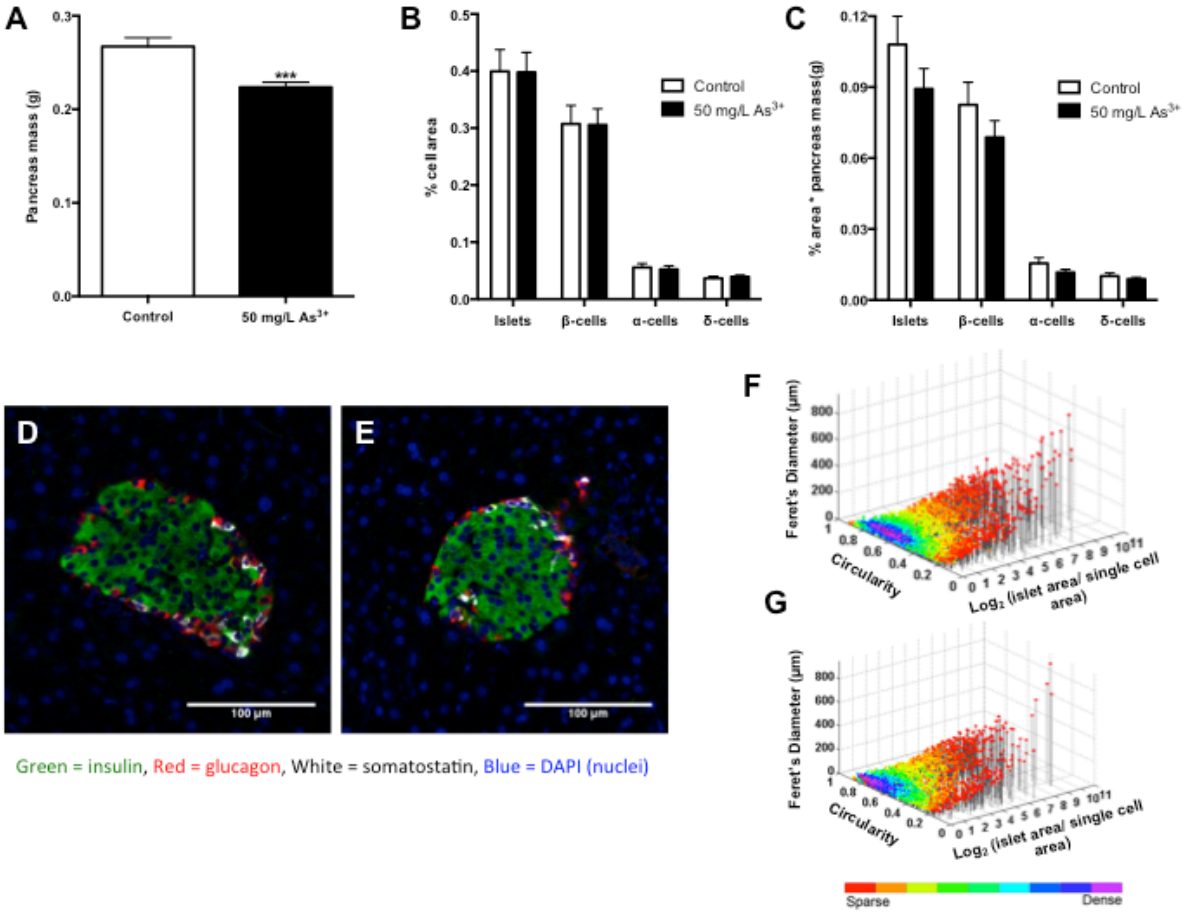

Figure 2.4.5: Arsenic does not alter relative pancreatic islet endocrine cell area
(a) Pancreas mass measured immediately following sacrifice. (b) Histological sections of pancreas from each mouse were immunostained and endocrine cell area (β-cell, α-cell, δ-cell, islet (additive)) was calculated relative to total area analyzed. (c) Islet mass as measured by % cell area multiplied by pancreas mass for each animal. (d) Representative image of a single islet from control mouse. (e) Representative image of a single islet from arsenic exposed mouse. (f) Scatter plot of individual islet morphology from all control mouse slides. (g) Scatter plot of individual islet morphology from all arsenic exposed mouse slides. n= 16 slides per group. Each slide is representative of one individual mouse. Figures show mean ± SEM. Statistics: ***$p<0.001$ by unpaired Student's t-test.

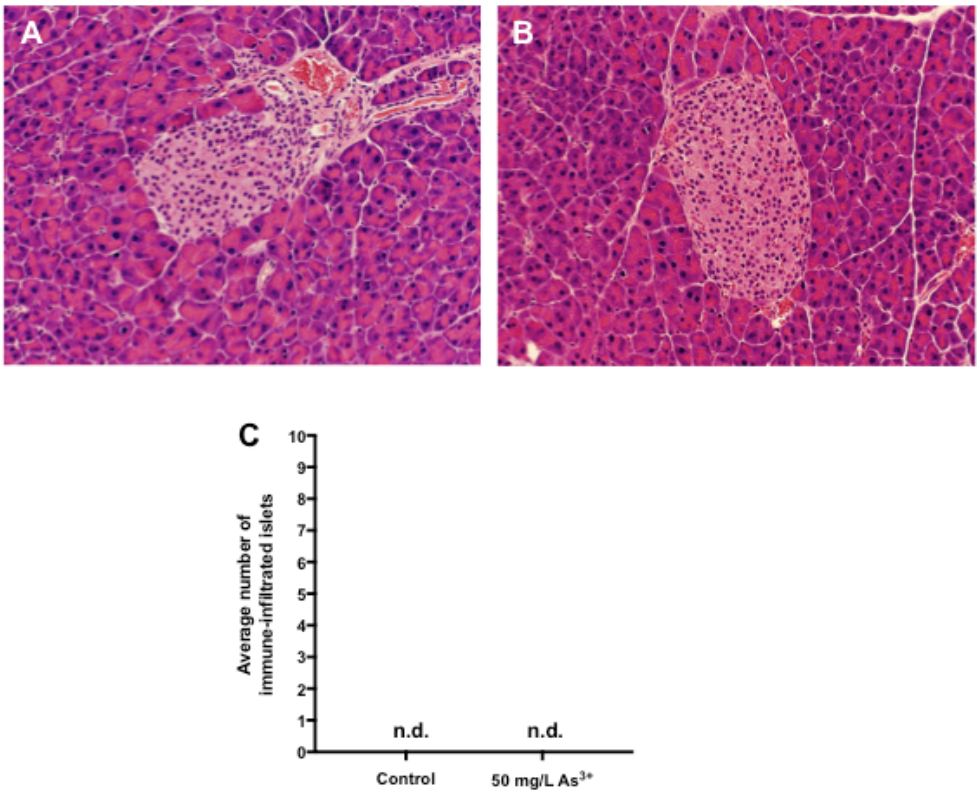

Figure 2.4.6: Arsenic does not have a significant effect on pancreatic islet inflammation
(a) Representative H&E image of a single islet from control mouse. (b) Representative H&E image of a single islet from arsenic-exposed mouse. (c) Quantification of islet immune cell infiltration. n= 16 slides reviewed per group. Each slide is representative of one individual mouse.

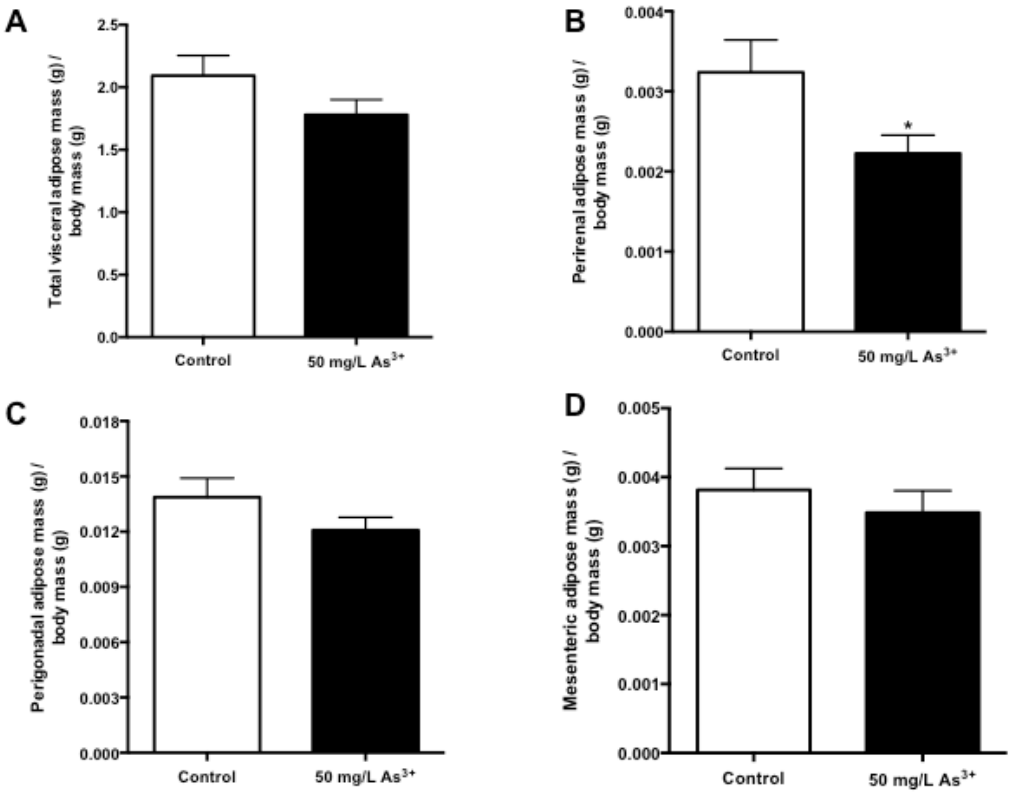

Figure 2.4.7: Arsenic exposure alters visceral adiposity in a depot-specific manner
(a) Visceral adipose depots (including (b) perigonadal, (c) perirenal, and (d) mesenteric depots) were collected upon sacrifice. Adipose depot mass is normalized to body mass for each individual mouse. n=16 mice per group. Figures show mean ± SEM. Statistics: *$p<0.05$ by unpaired Student's t-test.

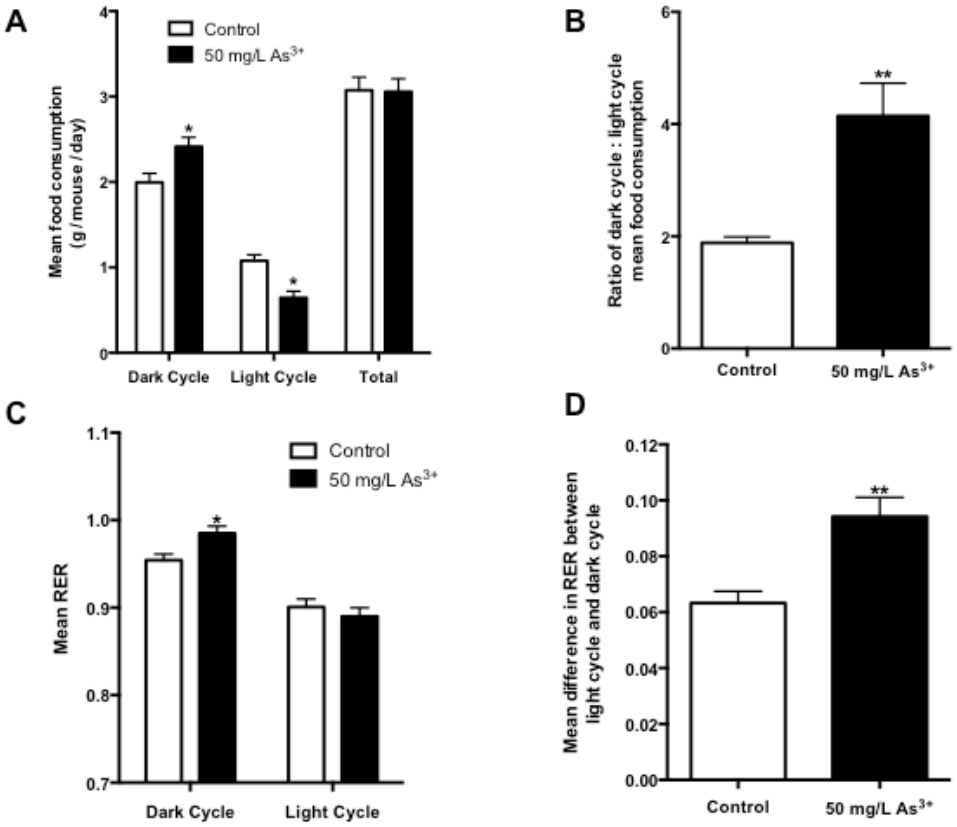

Figure 2.4.8: Arsenic exposure alters behavioral and metabolic circadian rhythms
(a) Mean food consumption was calculated for 2-3 alternating 12-hour light/dark cycles. (b) Mean ratio of dark cycle to light cycle food consumption was calculated per mouse. (c) Mean respiratory exchange ratio (RER) measured within 12-hour light/dark cycles. (d) Mean change in 12-hour RER between light and dark cycles for each individual mouse. n= 6 mice per group. Figures show mean ± SEM. Statistics: *p<0.05, **p<0.01, by unpaired Student's t-test.

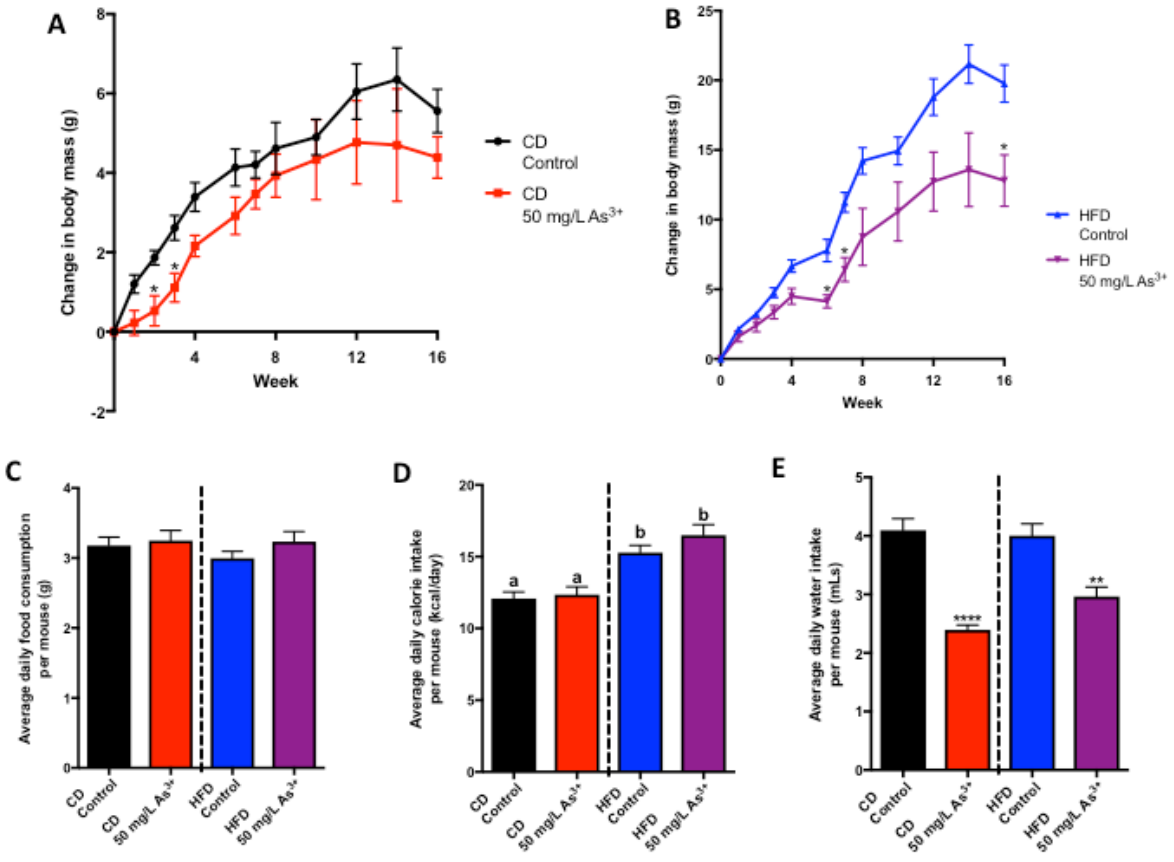

Figure 3.3.1: Arsenic exposure results in significantly lower weight gain in high-fat diet (HFD)-fed mice, despite no difference in caloric intake

(a) Weight gain over the course of the 16-week study for CD mice (n= 3-12 mice per group per time point). (b) Weight gain curve for HFD mice (n= 5-12 mice per group per time point). (c) Food intake as measured by net food consumption per cage on a weekly basis (n=6 cages per group). (d) Daily caloric intake based on caloric density for each diet (n=6 cages per group). (e) Water intake as measured by total consumption per cage on a weekly basis (n=6 cages per group). Data represented as mean ± SEM. Statistics: Unpaired Student's t-test (1a, 1b) or ordinary one-way ANOVA corrected for multiple comparisons (1c, 1d, 1e). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

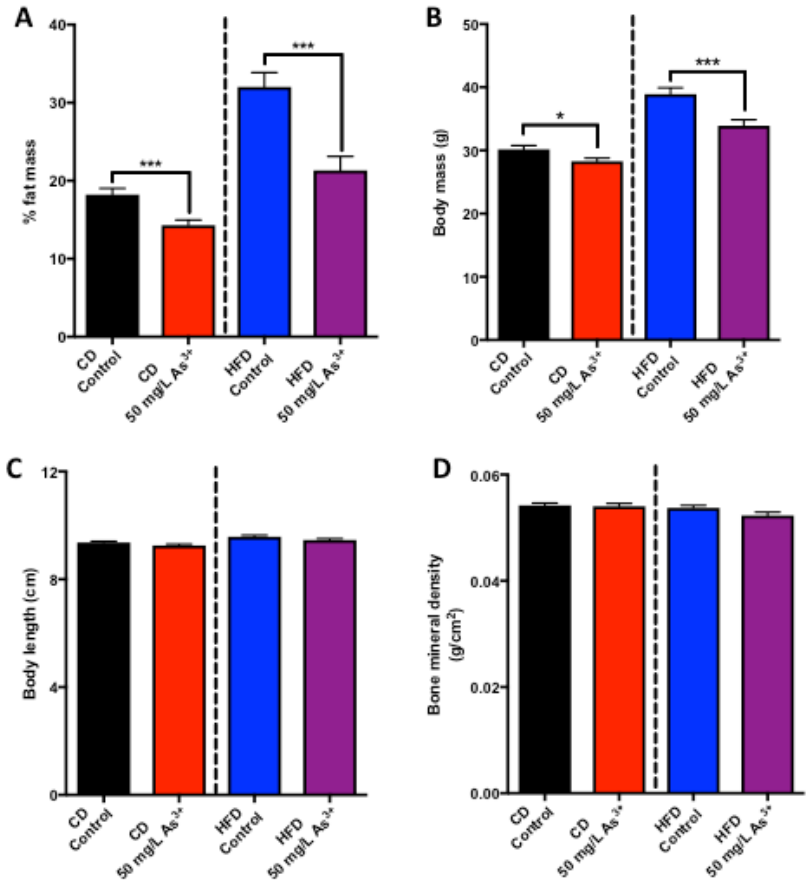

Figure 3.3.2: Mice exposed to arsenic exhibit decreased whole-body adiposity
(a) Total fat mass expressed as % of total body mass. (b) Total body mass as measured at the time of DEXA scan. (c) Total body length as measured at the time of DEXA scan. (d) Bone mineral density as measured by DEXA. Data represented as mean ± SEM. Statistics: Ordinary one-way ANOVA corrected for multiple comparisons, followed by unpaired Student's t-test for comparison of conditions within each dietary group. *p<0.05, **p<0.01, ***p<0.001.

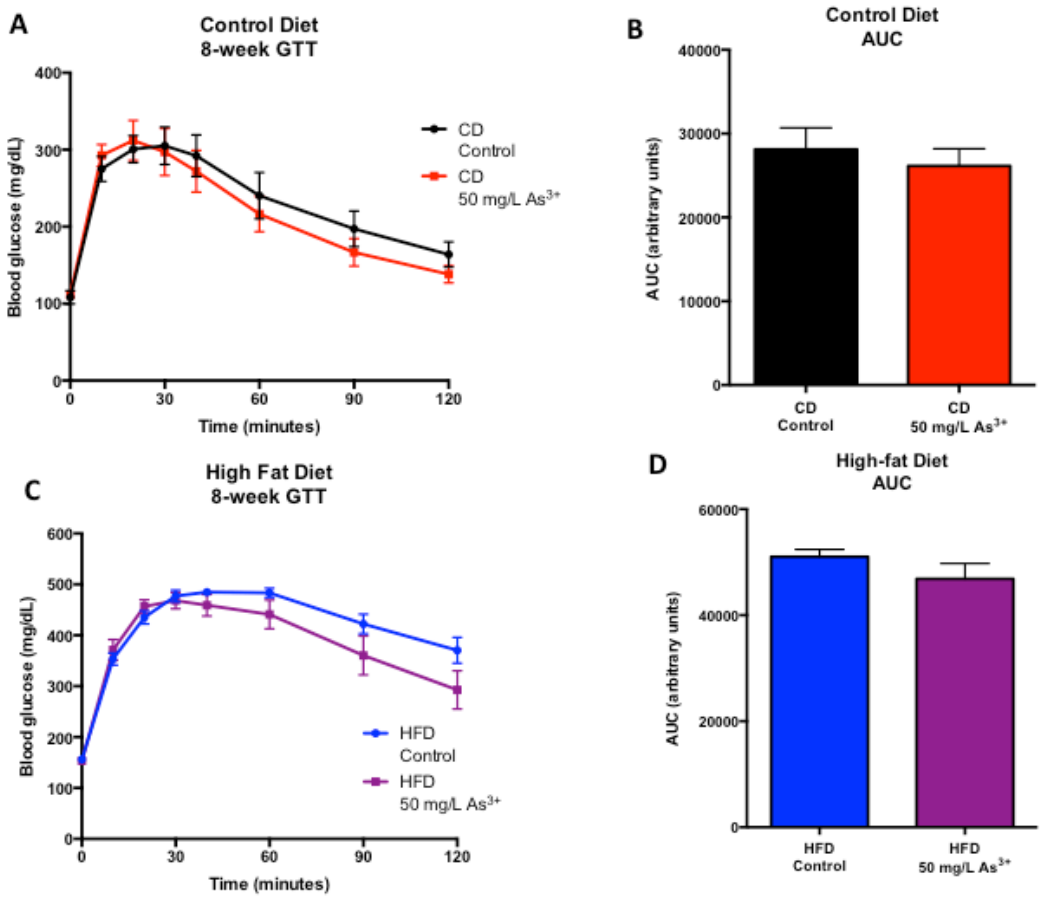

Figure 3.3.3: Arsenic exposure does not significantly alter glucose tolerance in either HFD- or CD-fed mice

(a) Glucose tolerance test (IP-GTT) performed following 8 weeks of exposure for CD-fed mice. (n=8-9 mice per group). (b) Area-under-the-curve (AUC) from the CD IP-GTT. (c) Glucose tolerance test (IP-GTT) performed following 8 weeks of exposure for HFD-fed mice. (n=11-12 mice per group). (d) Area-under-the-curve (AUC) from the HFD IP-GTT. Data represented as mean ± SEM. Statistics: Unpaired Student's t-test for AUC calculations, ordinary one-way ANOVA corrected for multiple comparisons for analysis of individual time-points over the course of the IP-GTT.

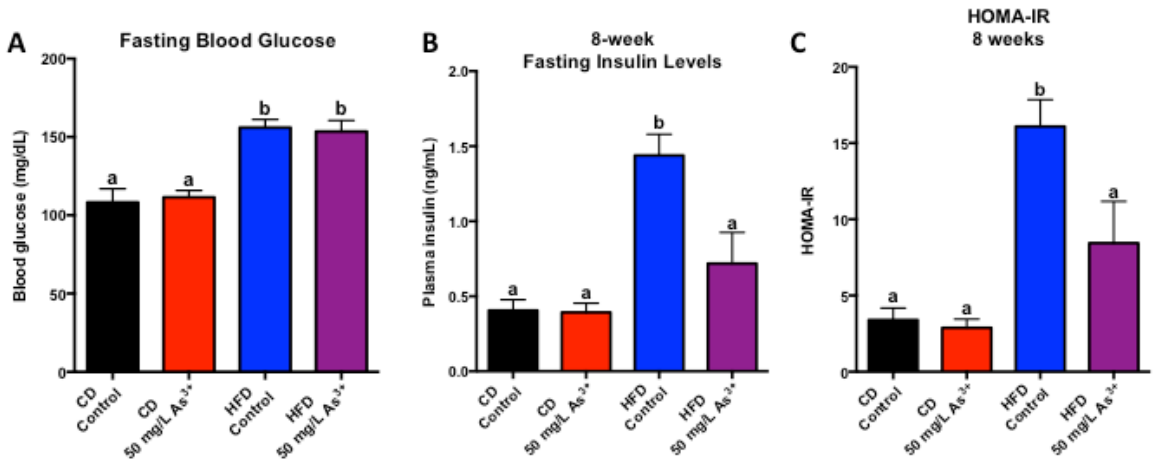

Figure 3.3.4: HFD feeding induces marked fasting hyperinsulinemia that is completely blunted by arsenic exposure

(a) Fasting blood glucose as measured following a 6-hour fast. (n=8-12 mice per group). (b) Fasting plasma insulin as measured following a 6-hour fast. (n=8-12 mice per group). (c) HOMA-IR values calculated using fasting blood glucose and fasting plasma insulin measures. Data represented as mean ± SEM. Statistics: Ordinary one-way ANOVA corrected for multiple comparisons. Groups sharing a letter are not statistically significantly different from one another ($p<0.05$ consider as statistically significant).

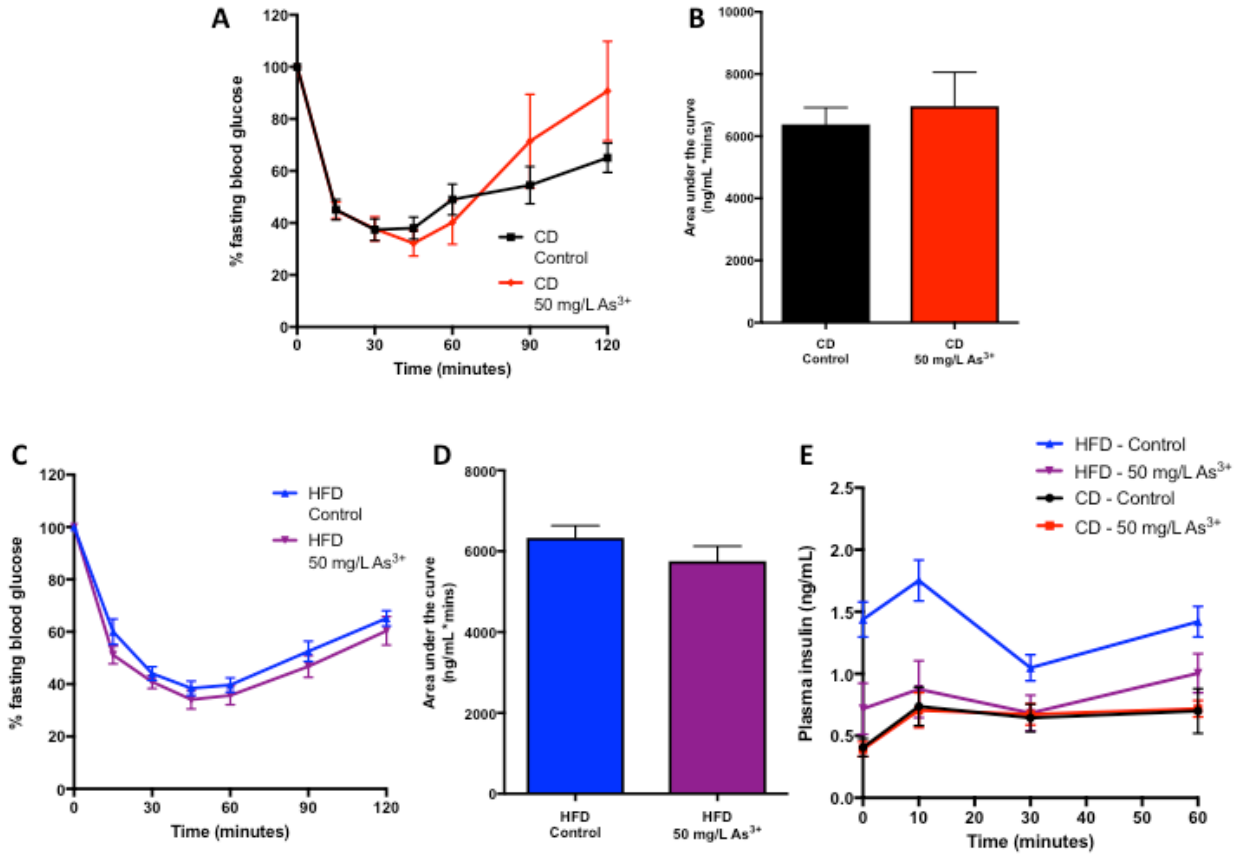

Figure 3.3.5: Arsenic-exposed, HFD-fed mice exhibit impaired insulin secretion dynamics despite exhibiting no difference in peripheral insulin sensitivity

(a) Intraperitoneal insulin tolerance tests (IP-ITT) performed following 7 weeks of exposure in CD-fed mice (n= 8-12 mice per group). (b) AUC from CD IP-ITT. (c) IP-ITT performed following 7 weeks of exposure in HFD-fed mice (n= 8-12 mice per group). (d) AUC from HFD IP-ITT. (e) Plasma insulin measured following the onset of glucose challenge during IP-GTT (n=8-12 mice per group). Data represented as mean ± SEM. Statistics: Unpaired Student's t-test for AUC calculations, ordinary one-way ANOVA corrected for multiple comparisons for analysis of individual time-points for IP-GTT plasma insulin levels.

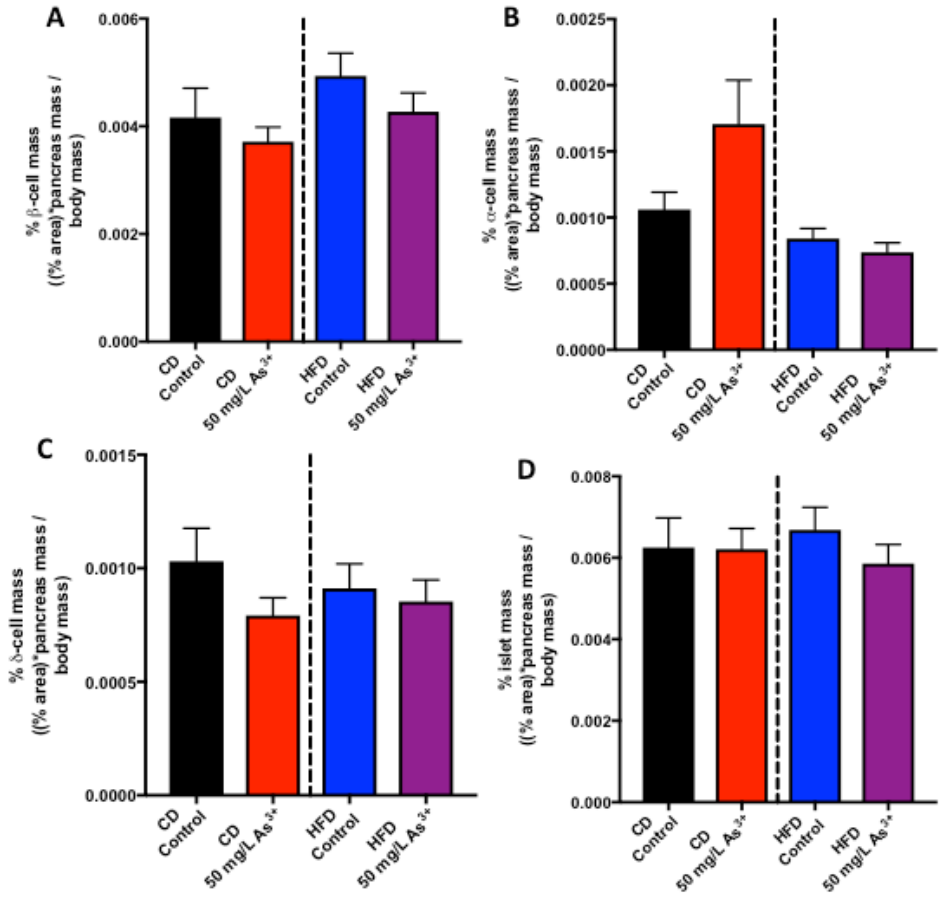

Figure 3.3.6: Differences in pancreatic endocrine cell mass do not fully explain differences in insulin secretion resulting from arsenic exposure

(a) Relative β-cell mass as calculated from IHC analysis of insulin(+) staining. (b) Relative α-cell mass as calculated from IHC analysis of glucagon(+) staining. (c) Relative δ-cell mass as calculated from IHC analysis of somatostatin(+) staining. (d) Relative beta-cell mass as calculated from IHC analysis of total endocrine cell staining area. Data represented as mean ± SEM. Statistics: Ordinary one-way ANOVA corrected for multiple comparisons, followed by unpaired Student's t-test within groups used for direct comparison of arsenic conditions within each dietary group. *$p<0.05$.

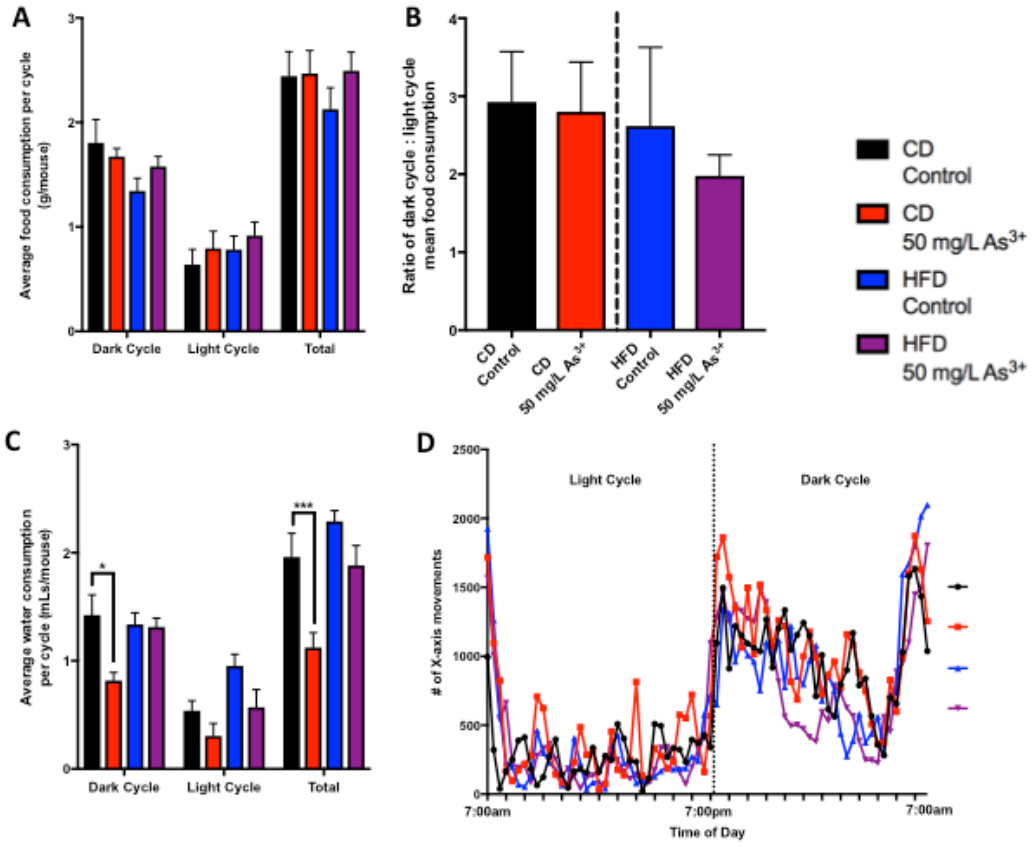

Figure 3.3.7: HFD-fed mice exhibit metabolic dysfunction compared to CD-fed mice and arsenic exposure does not significantly alter these metabolic parameters
(a) Total food consumption (g/mouse) per cycle. (b) Average dark:light cycle ratio of food consumption per mouse. (c) Total water consumption (mLs/mouse) per cycle. (d) Average number of x-axis movements (activity) tracked over time. Data represented as mean ± SEM. Statistics: Two-way ANOVA used to evaluate the effect of light cycle on each parameter. Ordinary one-way ANOVA corrected for multiple comparisons, followed by unpaired Student's t-test within light cycles used for comparison of conditions within each dietary group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

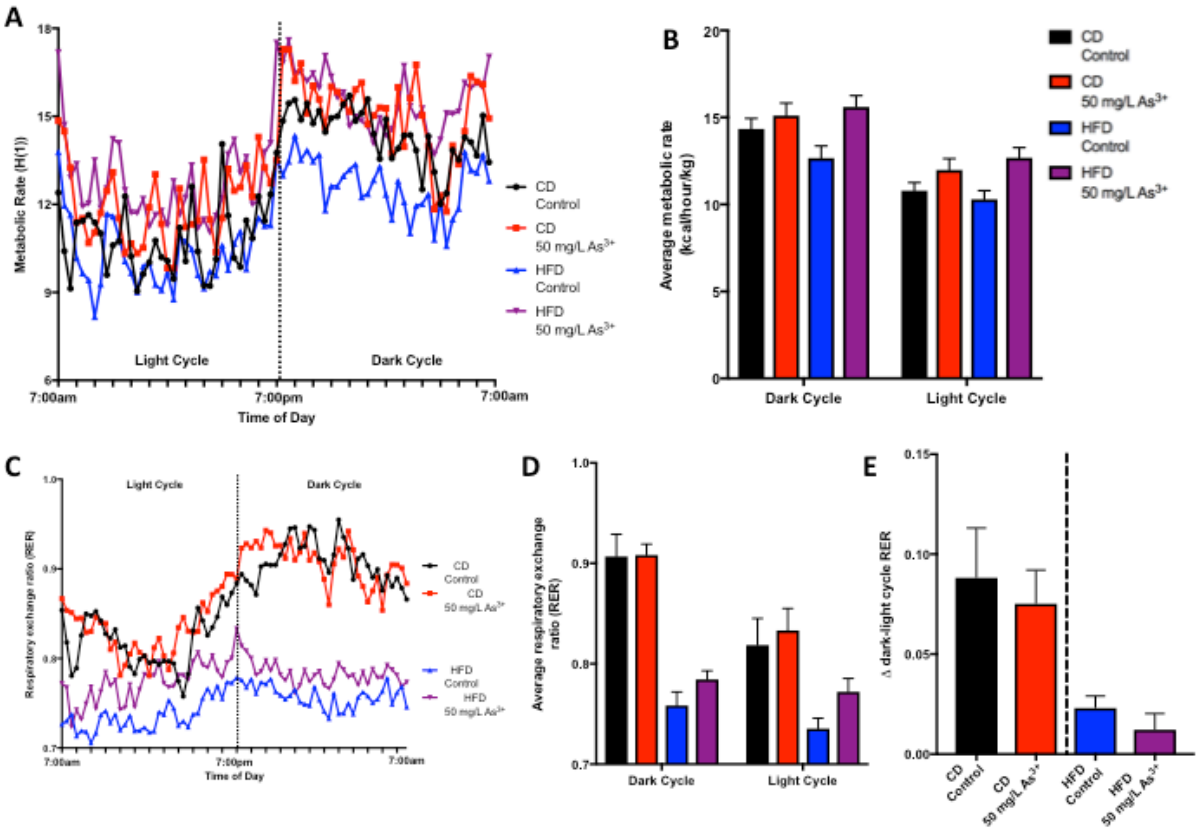

Figure 3.3.8: Circadian rhythmicity of energy substrate utilization is significantly altered by HFD consumption but not arsenic exposure

(a) Average metabolic rate tracked over time (b) Average metabolic rate (kcal expended/hr/kg body weight) per cycle. (c) Respiratory exchange ratio (RER) (VO_2/VCO_2) tracked over time. (d) Average respiratory exchange ratio (RER) (VO_2/VCO_2) per cycle. (e) Metabolic flexibility as measured by difference in average RER between light and dark cycles. Data represented as curves showing the means of each group only (7a and 7c) or mean ± SEM (7b and 7d). Statistics: Two-way ANOVA used to evaluate the effect of light cycle on each parameter. Ordinary one-way ANOVA corrected for multiple comparisons, followed by unpaired Student's t-test within light cycles used for comparison of conditions within each dietary group.

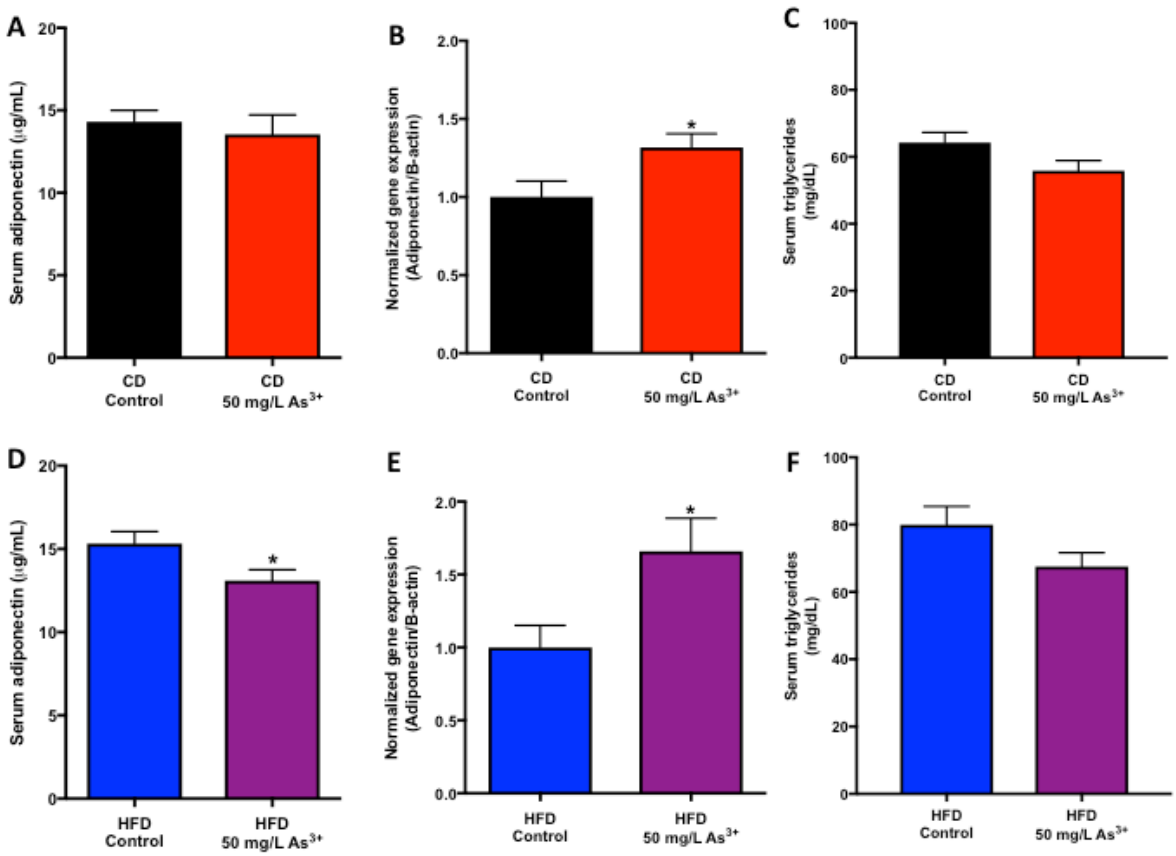

Figure 3.3.9: Adipose tissue pathology may contribute to the deterioration of metabolic health in arsenic-exposed mice

(a) Serum adiponectin levels from CD-fed mice as measured by ELISA (n=19 mice per group) (b) Relative adiponectin gene expression from perigonadal (visceral) adipose tissue as measured by qRT-PCR in CD-fed mice (n=6 mice per group) (c) Serum triglyceride levels in CD-fed mice as measure by colorimetric assay (n=19 mice per group). (d) Serum adiponectin levels from HFD-fed mice as measured by ELISA (n=20 mice per group) (e) Relative adiponectin gene expression from perigonadal (visceral) adipose tissue as measured by qRT-PCR in HFD-fed mice (n=6 mice per group) (f) Serum triglyceride levels in HFD-fed mice as measure by colorimetric assay (n=20 mice per group). Data represented as mean ± SEM. Statistics: Unpaired Student's t-test to directly compare results within each diet (CD or HFD) group, *$p<0.05$.

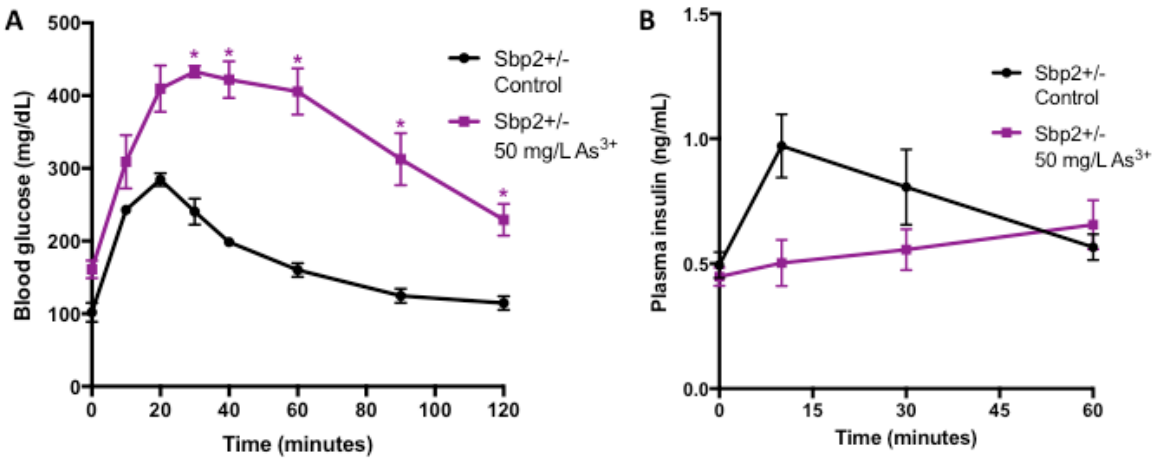

Figure 4.1.2: Arsenic-induced glucose intolerance is exacerbated in global Sbp2 heterozygous (Sbp2$^{+/-}$) mice fed on a low-selenium diet

(a) Glucose tolerance test (IP-GTT) performed following 8 weeks of exposure for Sbp2$^{+/-}$ mice fed on a low-selenium diet (n=3 mice per group). (b) Plasma insulin levels as measured over time for the IP-GTT (n=3 mice per group) Data represented as mean ± SEM. Statistics: Ordinary one-way ANOVA corrected for multiple comparisons for analysis of individual time-points for IP-GTT glucose levels (4.1.2a) and plasma insulin levels (4.1.2b).

Appendix B: Tables

Macronutrient Comparison	Facility Chow (TD.2918) Natural Ingredients	Control (CD) (TD.97184) Purified Diet	High-fat (HFD) (TD.160079) Purified Diet
Carbohydrates (% kcals from)	58	64	21
Fat (% kcals from)	18	17	60
Protein (% kcals from)	24	19	18
Caloric density (total kcal/g)	3.1	3.8	5.1

Table 3.2.2a: Comparison of macronutrient content in experimental diets
All diets were obtained from Envigo. Nutritional informational provided by the manufacturer.

Micronutrient Comparison	Facility Chow (TD.2918) Natural Ingredients	Control (CD) (TD.97184) Purified Diet	High-fat (HFD) (TD.160079) Purified Diet
Copper (Cu) (mg/kg diet)	16	6.2	8.5
Iron (Fe) (mg/kg diet)	200	37.1	50.8
Iodine (I) (mg/kg diet)	6	0.21	0.28
Manganese (Mn) (mg/kg diet)	100	10.5	14.5

Table 3.2.2b: Comparison of select micronutrient content in experimental diets
All diets were obtained from Envigo. Nutritional informational provided by the manufacturer.