Genetic Sensitivity to Mitochondrial Toxicity

by

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Heather M. Stapleton

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in Environment in the Graduate School
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ABSTRACT

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Abstract

Mitochondria are the main cellular producers of ATP, and play key roles in cellular signaling and apoptosis. Mitochondria also contain their own genomes (mtDNA), which encode 13 subunits of the electron transport chain (ETC), 22 tRNAs, and 2 rRNAs, making mtDNA integrity critical to both mitochondrial and organismal health. Mitochondria are dynamic organelles that fuse and divide to maintain mitochondrial shape, number, and size. However, mitochondrial fission and fusion also play a major role in the mitochondrial stress response. For example, mildly damaged mitochondria can fuse with healthy mitochondria allowing contents to mix, resulting in the generation of healthy mitochondria, which is known as functional complementation. Alternatively, when mitochondria become damaged beyond repair, they are targeted for autophagosomal degradation, or mitophagy. The overall importance of fission, fusion, mitophagy, and mtDNA is demonstrated by the fact that deficiencies in these processes and mtDNA content cause human disease. Interestingly, the age of onset, and severity of clinical manifestations of mitochondrial disease vary from patient to patient, even in individuals harboring identical mutations. These observations suggest a role for the environment in the development and progression of certain mitochondrial diseases; however, the relationship remains poorly understood.
To investigate the role of environmental toxicants in the development, progression, and exacerbation of mitochondrial disease I have taken two approaches using the *in vivo* model organism *Caenorhabditis elegans*. First, ten known and suspected mitochondrial toxicants (2,4-dinitrophenol (DNP), acetaldehyde, acrolein, aflatoxin B₁ (AfB₁), arsenite, cadmium, cisplatin, doxycycline, paraquat, rotenone) were screened for exacerbation of larval growth delay in wild-type, fission-, fusion-, and mitophagy-deficient nematodes using the COPAS Biosort. Second, a *C. elegans* model of mtDNA depletion was developed using chronic low-dose ethidium bromide exposure. Five toxicants (AfB₁, arsenite, paraquat, rotenone, ultraviolet C radiation (UVC)) were tested for exacerbation of mitochondrial function (assessed via changes in steady-state ATP levels) in nematodes with reduced mtDNA content. Mitochondrial health was then further assessed for some of the identified gene-environment interactions. Mitochondrial respiration was measured using the Seahorse XF-24 Extracellular Flux Analyzer, while steady-state ATP levels were assessed using transgenic luciferase expression nematodes and traditional extraction protocols. Gene expression, mtDNA, and nuclear DNA copy number were assessed using real-time PCR, while enzyme activity was assessed using microplate reader-based assays.

Results from the fission, fusion, and mitophagy toxicant screen revealed that fusion-deficient nematodes were sensitive to a variety of toxicants (DNP, AfB₁, arsenite, cisplatin, paraquat, rotenone), while *pink-1* mitophagy-deficient nematodes were
sensitive to rotenone, and fission- and pdr-1 mitophagy-deficient nematodes were only mildly sensitive to paraquat, and rotenone, respectively. As mitochondrial disease is rare, but chronic arsenite exposure is widespread, we further investigated the mechanisms underlying arsenite sensitivity in fission- and fusion-deficient nematodes. Although not sensitive in the larval growth assay, fission-deficient nematodes were sensitive to arsenite later in life in both reproduction and lethality assays. Seahorse and ATP analysis revealed that arsenite disrupts mitochondrial function in fusion-deficient nematodes at multiple life stages (L4, 8- and 12-days of age), while increasing mitochondrial function in 8-day old wild-type nematodes, and has minimal effect on mitochondrial function in fission-deficient nematodes. Lastly, arsenite inhibited both pyruvate and isocitrate dehydrogenase activity in fusion-deficient nematodes, suggesting a disruption of pyruvate metabolism and Krebs cycle activity underlie the observed mitochondrial dysfunction. These results suggest that deficiencies in mitochondrial fusion may sensitive individuals to arsenite toxicity.

Lastly, I have found that reducing mtDNA content 35-55% only mildly sensitized nematodes to certain secondary toxicant exposures, including UVC and arsenite. Alternatively, reduced mtDNA content did not sensitize nematodes to acute or chronic paraquat or AfB1 exposure, and provided resistance to rotenone. However, we also found that EtBr can induce cytochrome P450s (CYPs), which play a major role in rotenone metabolism; thus, it is likely that induction of CYPs and not reduced mtDNA
content is responsible for rotenone resistance. These results suggest that individuals with reduced mtDNA content may be sensitive to certain toxicant exposures, but also highlight the robust mechanism that exist to maintain the integrity of mitochondria and mtDNA.

Collectively, these results suggest individuals suffering from mitochondrial disease caused by mutations in mitochondrial fission, fusion, or mitophagy genes, or by depletion of mtDNA, may be especially sensitive to certain environmental toxicant exposures, including arsenic. Arsenic’s pervasive contamination of drinking water results in chronic exposure for over 100 million people worldwide; thus, dramatically increasing the probability of exposure for individuals suffering from mitochondrial disease, and warrants further investigation in the human populous.
Dedication

This work is dedicated to my Aunt Linda. You always supported and encouraged me. I love and miss you, and hope I have made you proud.
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1. Introduction

1.1 Mitochondria

Mitochondria, best known for their role in generating cellular ATP via oxidative phosphorylation (OXPHOS), are dynamic organelles encapsulated by a phospholipid-rich double membrane. The outer mitochondrial membrane (OMM) is permeable to small metabolites and ions due to the presence of voltage-dependent anion channels (VDACs), while the inner mitochondrial membrane (IMM) is impermeable to most ions and metabolites without the aid of specialized transporters (1). The five complexes of the electron transport chain (ETC) are located on the IMM, which forms invaginations into the mitochondrial matrix, known as cristae, allowing for increased surface area and thus a greater capacity for proton pumping (2). Mitochondria also contain their own circular genomes, which encode 13 critical subunits of the ETC (discussed further below) (3); however, the vast majority of mitochondrial proteins are encoded in the nuclear genome and must be imported into the mitochondria to maintain proper function.

In addition to OXPHOS, mitochondria house many other important biochemical pathways, such as the Krebs cycle and fatty acid oxidation, and generate many metabolites required for anabolic processes such as gluconeogenesis, nucleotide, fatty acid, and amino acid synthesis, thus making mitochondria the nexus of intermediary metabolism (4). However, mitochondria also play crucial roles in other important biological processes such as apoptosis and signaling (5, 6). Given these diverse functions
it is not surprising that mitochondrial dysfunction has been implicated in numerous human diseases ranging from cancer, to diabetes, to neurodegeneration (7, 8).

1.2 Intermediary metabolism and oxidative phosphorylation

Mitochondria contain the enzymatic machinery to complete the final oxidation steps of sugars, fatty acids and proteins to generate energy in the form of ATP. Sugars are broken down via glycolysis in the cytosol and enter mitochondria as pyruvate. In the mitochondrion, pyruvate dehydrogenase decarboxylates pyruvate forming acetyl-CoA, which can then enter the Krebs cycle. Alternatively, fatty acids are broken down in the mitochondria via fatty acid β-oxidation to acetyl-CoA, while various enzymes exist to catabolize amino acids into Krebs cycle intermediates (4).

The Krebs cycle lies at the center of intermediary metabolism, as it not only provides reducing equivalents for the ETC, but also provides intermediates that can be used for the synthesis of macromolecules (lipids, nucleotides) and signaling. For example, mitochondrial citrate can be converted to acetyl-CoA in the cytosol and used for histone acetylation (4, 5). The Krebs cycle is composed of eight enzymatic reactions that generate three molecules of nicotinamide adenine dinucleotide (NADH) and one molecule of Flavin adenine dinucleotide (FADH$_2$) upon completion of the cycle (9).

The electron transport chain is composed of five complexes located on the IMM. Complex I (CI), also known as NADH dehydrogenase, accepts two electrons from NADH, which are passed to the lipid soluble electron carrier coenzyme Q (Q). The flow
of electrons through CI is linked to the pumping of four protons across the IMM into the inter membrane space (IMS). ETC CII, or succinate dehydrogenase, accepts electrons from \( \text{FADH}_2 \), and also passes them to \( Q \); however, no proton pumping is associated with the flow of electrons through CII. Reduced \( Q \) freely diffuses through the IMM and transfers electrons to ETC CIII, or cytochrome c reductase, which then passes electrons one at a time to the electron carrier cytochrome c, resulting in the pumping of two additional protons. Finally, cytochrome c donates electrons to ETC CIV, or cytochrome c oxidase, which passes the electrons to the final electron acceptor, molecular oxygen, resulting in the formation of \( \text{H}_2\text{O} \) and the pumping of an additional four protons. Proton pumping by the ETC results in the formation of a proton gradient across the IMM that is used to power ATP synthase. As protons flow through ATP synthase the released energy is used to generate ATP from ADP and phosphate (9, 10).

The mitochondrial ETC is also a major site of reactive oxygen species (ROS) production. ROS is generated when electrons leak from CI or CIII and react with oxygen to form superoxide (11). Both ETC CI and CIII can leak electrons into the mitochondrial matrix, while only CIII can leak electrons into the IMS (12). Mitochondrial ROS can damage macromolecules such as lipids, proteins, and mtDNA [Reviewed in (13-15)] causing mitochondrial dysfunction, which in turn can lead to further ROS production and further mitochondrial dysfunction, in a process known as the vicious cycle (16).

Although generation of mitochondrial ROS has been implicated in many pathologies,
mitochondria contain robust mechanisms to detoxify ROS. Mitochondrial manganese superoxide dismutase can dismutate two superoxide anions to hydrogen peroxide (H$_2$O$_2$), which in turn can be broken down to water via catalase or conjugated to glutathione for excretion (17). Additionally, H$_2$O$_2$ can reactive with iron to form the highly reactive hydroxyl radical via Fenton type chemistry. Finally, ROS can activate mitochondrial uncoupling proteins that dissipate the proton gradient, which serves to help limit further ROS production through a negative feedback loop (18).

Alternatively, low-level ROS can function as signaling molecules, affecting numerous biological processes, including immune response, stem cell differentiation and proliferation, and hypoxic signal transduction (19). Protein function is altered through the reversible oxidation of redox-sensitive thiol groups by hydrogen peroxide (20), which in turn can alter cross-talk between the mitochondria and nucleus. Interestingly, low-dose toxicant exposures can have beneficial effects (a phenomenon known as hormesis (21)), including upregulation of stress response pathways. For example, low-dose paraquat and arsenite can induce transient bursts of mitochondrial ROS, leading to increased stress resistance and lifespan extension in nematodes (22, 23).

### 1.3 Mitochondrial DNA

In humans, the mitochondrial genome (mtDNA) is 16,569 base pairs in size and encodes 13 critical subunits of the ETC, 22 tRNAs, and 2 rRNAs (3). Depending upon cell type, energetic demands, and developmental stage, the number of mtDNA copies
per cell can range from several hundred to 10,000 or more copies per cell (24). Unlike nuclear DNA, mtDNA lacks protective histones, which is thought to increase the vulnerability of mtDNA to damage. Instead, mtDNA is packaged into a DNA-protein structure called the nucleoid (25, 26). Each nucleoid has been reported to contain 2-10 genomes, although more recent work has suggested that 1-2 mtDNAs/nucleoid may be common (27). Nucleoids are anchored to the IMM in close proximity to the ROS-generating ETC, making mtDNA susceptible to oxidative damage (15, 16). In addition to mtDNA, the nucleoid comprises numerous proteins, including the DNA helicase twinkle, the Krebs cycle enzyme aconitase, mitochondrial transcription factor a (TFAM), as well as several other IMM proteins (28, 29). Interestingly, nucleoids are not static, and emerging evidences suggests nucleoids can be remodeled in response to DNA intercalating agents (30, 31) and oxidative stress (32). Furthermore, nucleoid remodeling may help to protect mtDNA from damage or depletion.

1.4 Fission, fusion, and mitophagy

Mitochondria are dynamic organelles that fuse and divide in response to diverse stimuli to regulate mitochondrial shape, number, and size (Figure 1). Mitochondrial fission and fusion are regulated by large guanosine triphosphatases in the dynamin family, which are well conserved between mammals, flies, and C. elegans (33). Dynamin related protein 1 (DRP1) mediates mitochondrial fission. Cytosolic DRP1 is recruited from the cytosol and forms spirals around the mitochondria, often times at sites of
endoplasmic reticulum-mitochondria contact (34), and upon GTP hydrolysis constricts to drive scission of the mitochondria. Alternatively, OMM fusion is mediated by mitofusin 1 and 2 (MFN1 and MFN2), while IMM fusion is mediated by OPA1 (35). In addition to regulating mitochondrial morphology, fission and fusion also play a role in the regulation of mitochondrial metabolism, as hyperfused mitochondria tend to be more metabolically active than fragmented mitochondria (36, 37).

Mitochondrial fission and fusion also play important roles in the mitochondrial stress response. For example, mitochondria carrying low levels of damage can fuse with a healthy mitochondria allowing contents to mix, resulting in the formation of a single healthy mitochondria that can then divide into multiple healthy daughter mitochondria in a process known as functional complementation (38). However, severely damaged mitochondria that cannot maintain mitochondrial membrane potential are not competent to rejoin the mitochondrial network. Instead, loss of membrane potential triggers the proteolytic cleavage of the IMM fusion protein OPA1, thus preventing fusion and poisoning of the mitochondrial network (39). Damage mitochondria are then eliminated via targeted autophagy, or mitophagy, as the PTEN-induced putative kinase 1 (PINK1) accumulates on the OMM and phosphorylates OMM proteins. The ubiquitin ligase parkin (PARK2) is then recruited to the OMM and ubiquitinates mitochondrial proteins targeting the mitochondria for degradation (40, 41). Mitochondrial fission, fusion, and mitophagy are tightly linked processes. Prior to mitophagy, mitochondria
typically fragment, as hyperfused mitochondria tend to be recalcitrant to mitophagy and autophagosomal degradation, while fragmented mitochondria are more readily degraded (35).

The overall importance of mitochondrial dynamics is demonstrated by the fact that deficiencies in these processes are associated with human disease. For example, mutations in PINK1 and PARK2 are associated with early-onset Parkinson’s disease (42), while mutations in OPA1 cause dominant optic atrophy (43), and mutations in MFN2 cause Charcot-Marie Tooth neuropathy type 2A (44). Alternatively, mutations in DRP1 are not typically associated with human disease, although a few case reports of neurodegeneration have been reported (45, 46).

In addition to controlling mitochondrial dynamics and morphology, fission, fusion, and mitophagy genes can play additional roles in modulating mitochondrial health. For example, OPA1 plays a role in cristae maintenance and mtDNA stability (47), while PINK1 can regulate ETC CI activity through phosphorylation (48), and MFN2 can tether mitochondria to the endoplasmic reticulum allowing calcium and redox signaling to occur between the two organelles (49, 50). MFN2 is also a target of PINK1 phosphorylation, and phosphorylation of MFN2 is required for the efficient recruitment of parkin and mitophagy in some in vitro models (51).
1.5 Mitochondrial disease

Mitochondrial disease, characterized by impaired energy production, is caused by mutations in both mitochondrial and nuclear encoded subunits of the electron transport chain (52). Disease caused by mutations in nuclear encoded OXPHOS subunits follow Mendelian rules of inheritance, while disease caused by mutations in mtDNA follow population level genetics for the reason that mtDNA exists in hundreds to thousands of copies per cell. Somatic or maternally inherited mtDNA mutations result in multiple mtDNA variants per cell (heteroplasmy), resulting in a threshold effect, in which 60-90% of mtDNA must be mutated or damaged prior to pathogenesis. Interestingly, pathogenic mtDNA mutations are not rare, as 1 in 200 individuals are estimated to harbor pathogenic mtDNA mutations below the pathogenic threshold (53). Although random genetic drift can cause clonal expansion of mutated mtDNA variants (54, 55), the precise mechanism underlying expansion of mtDNA mutations remain unclear.

In addition to mutations, depletion of mtDNA can also cause a severe group of autosomal recessive disorders known as mtDNA depletion syndrome [reviewed in (56, 57)]. MDS is one of the most common childhood mitochondrial disorders, and may account for up to 50% of all unexplained cases of respiratory chain disease (58). MDS manifests in either single or multiple organs, including the brain, liver, kidney, muscle, or intestine. MDS is characterized by a severe loss of mtDNA (up to 98%) caused by
mutations in nuclear encoded genes that function in mtDNA maintenance or mitochondrial nucleotide synthesis, resulting in impaired ATP production due to insufficient expression of respiratory chain complexes (59).

The molecular mechanisms underlying MDS are beginning to be elucidated. As replication of mtDNA is independent of the cell cycle, the mitochondrial deoxyribonucleoside triphosphate (dNTPs) pool is not maintained via the de novo synthesis pathway, which is regulated via the cell cycle; instead, mitochondrial dNTP pools are maintained via the nucleoside salvage pathway. Thymidine kinase 2 (TK2), deoxyguanosine kinase (DGUOK), ribonucleotide reductase (RRM2B), thymidine phosphorylase (TP) and subunits of succinyl CoA ligase (SUCLA2, SUCLG1) participate in the nucleoside salvage pathway, thus mutations in these genes result in reduced dNTP pools and depletion of mtDNA. Alternatively, POLG encodes the catalytic subunit of the sole replicative mtDNA polymerase, while C10orf2 encodes the DNA helicase twinkle. Both genes are required for mtDNA replication, and mutations in either gene can result in mutagenesis and insufficient mtDNA synthesis leading to depletion of mtDNA (56, 57, 59). However, mutations in other genes, such as the mitochondrial fusion genes OPA1 and MFN2, can also cause mtDNA depletion (60-62). Furthermore, exposure to nucleoside reverse transcriptase inhibitors (NRTIs), which are used to prevent the transmission of HIV from mother to child, can also reduce mtDNA copy number through inhibition of mtDNA polymerase gamma (63). NRTI-induced depletion
of mtDNA tends to occur in energetically demanding tissues such as the brain, heart and muscle, and although mtDNA copy number typically returns to control levels following drug cessation, several studies have reported persistent reductions (64, 65).

1.6 Mitochondria are a target of environmental toxicants

Mitochondria and mtDNA are vulnerable to toxicity induced by drugs and toxicants for a variety of reasons [reviewed in (66)]. First, the mitochondrial phospholipid-rich double membrane can attract lipophilic toxicants such as polycyclic aromatic hydrocarbons (PAHs) and aflatoxins (AfB) (67, 68). Once in the mitochondria, these relatively unreactive toxicants can be bioactivated to their reactive, DNA damaging forms via mitochondrial localized cytochrome P450s, and cause bulky, polymerase-stalling DNA lesions (69-71). As mitochondria lack the nucleotide excision repair pathway (72), which is responsible for the repair of bulky lesions, mtDNA damage caused by PAHs, AfBs, and other drugs and toxicants such as cisplatin and acrolein are irreparable in the mitochondrial genome (73, 74). These persistent, bulky, toxicant-induced lesions have been hypothesized to induce mtDNA mutations that can contribute to mitochondrial dysfunction and disease. However, in a recent study, benzo(a)pyrene (BaP), which induces mutations in the nuclear genome, was not detectably mutagenic in the mitochondrial genome in several cell types in mice (75). Instead, we have previously shown that irreparable mtDNA damage is slowly removed
through the recycling of damaged genomes by the processes of mitochondrial fission, fusion, mitophagy and autophagy (76, 77).

Secondly, due to molecular mimicry, metals such as lead, cadmium, manganese, and mercury may enter and accumulate in mitochondria through the calcium transporter (78-81). Once in the mitochondria, these metals, many of which have high affinity for protein thiol and selenocysteine groups, can inhibit enzymes and induce mitochondrial dysfunction. For example, cadmium can disrupt mitochondrial respiration through inhibition of ETC CIII and citrate synthase in several in vivo models (82-84). Furthermore, disruption of the ETC can generate ROS, which can in turn further damage macromolecules such as mtDNA, protein, and lipids. Also of concern is mtDNA, which is anchored to the IMM in close proximity to the ETC, and thus is highly susceptible to oxidative damage; however, robust activity of the base excision repair pathway (BER) in mitochondria can limit damage (85).

Finally, proton pumping across the IMM by the ETC gives the mitochondrial matrix a negative charge and slightly alkaline pH that can attract positively charged metals and lipophilic amphiphilic xenobiotics, many of which are known mitochondrial toxicants (66, 86). For example, the redox cycler paraquat can accumulate in mitochondria, and has been shown to preferentially induce oxidative mtDNA damage in C. elegans (87).
Given that many toxicants can cause mitochondrial dysfunction, and mitochondrial dysfunction can cause disease, it is not surprising that mitochondrial toxicant exposure has been linked to certain human diseases. For example, exposure to paraquat and rotenone (ETC CI inhibitor) has been linked to the development of Parkinson’s disease in farmworkers (88).

1.7 Mitochondrial gene x environment interactions

Much of what we know about mitochondrial gene-environment interactions comes from the pharmaceutical industry. This is because chemicals that are intended for industrial use are not nearly as well studied for toxicity as are drugs. Two of the best-characterized drugs known to have adverse effects when administered to individuals with mitochondrial deficiencies are valproate (89, 90) and gentamycin (91). Gentamycin is an aminoglycoside antibiotic used in the treatment of bacterial infections. Although not easily metabolized, gentamycin is removed from the majority of tissues by glomerular filtration (92); however, gentamycin can accumulate in the inner ear and cause deafness (93). In context of certain point mutations in mitochondrial 12s rRNA, which render the 12s rRNA more similar in structure to bacterial 16s rRNA, ototoxicity is exacerbated due to impairment of mitochondrial protein synthesis (94). Another example is valproate, widely used for the treatment of epilepsy. Because valproate is a simply fatty acid, it can be metabolized via mitochondrial fatty acid β-oxidation (FAO),
and valproate and its metabolites can directly inhibit certain FAO enzymes leading to liver failure in patients with inborn errors in metabolism (90, 95, 96).

Although valproate and gentamycin are the best-characterized mitochondrial-gene environment interactions, emerging evidence suggests patients suffering from CMT2A and DOA may be especially sensitized to certain neurotoxic agents, which may be due to the dual neurodegenerative nature of these diseases and toxic agents (97). For example, ethambutol (EMB) is an antimicrobial agent used in the treatment of tuberculosis, and its use is associated with optic neuropathies in 2% of patients (98, 99). Recently, treatment with EMB was reported to cause optic atrophy in a patient harboring a mutation in OPA1; however, no visual impairment was present prior to EMB treatment (100). Furthermore, EMB treatment has also been reported to cause optic atrophy, worsening of neuropathy, and vocal cord paralysis in a patient suffering from CMT2A caused by a mutation in MFN2 (101). Although these two case reports suggest an interaction between mitochondrial fusion and EMB, further mechanistic work is required.

1.8 The C. elegans model

*Caenorhabditis elegans* is a free-living nematode that inhabits decaying leaf litter on the forest floor (102). First developed by Sydney Brenner over 50 years ago as model for studying developmental and neurobiology, *C. elegans* has since emerged as an *in vivo* model for toxicologists [reviewed in (103-105)]. *C. elegans*’ small size (1mm in length),
short developmental period (1st larval stage to gravid adult in 72h), short lifespan (2-3 weeks), and high reproductive rate (300 offspring per wild-type nematode), make *C. elegans* amenable to low-cost, high-throughput toxicity screening (106, 107).

Furthermore, *C. elegans* mitochondrial and nuclear genomes have both been sequenced, and share a high degree (~80%) of homology with humans. Many of the same reasons that make nematodes an attractive model for toxicity testing also make them an attractive model for studying mitochondrial toxicity and disease [reviewed in (108, 109)]. Mitochondrial biology is highly conserved between humans and nematodes. *C. elegans*’ mitochondrial genome is 13,769bp in size and encodes 12 subunits of the ETC (ATP8 is unconfirmed, but also likely present (110)), 22 tRNAs and 2 rRNAs (111). Nuclear encoded subunits of the ETC are also well conserved between humans and *C. elegans* (112), as is overall ETC function (113). Finally, many of the pathways of intermediary metabolism, including glycolysis, gluconeogenesis, Krebs cycle, and fatty acid oxidation are highly conserved in nematodes (113-116). Given that mitochondrial function is highly conserved, and that 80% of genes associated with inborn errors in metabolism in humans have *C. elegans* orthologs (117), it is not surprising that *C. elegans* have been used as a model for studying mitochondrial disease (108), toxicity (109), neurodegeneration (118, 119), and lipid metabolism disorders (115).

Finally, developmental stage- and tissue-specific differences in mitochondrial function are preserved in *C. elegans*. This is advantageous for studying mitochondrial
biology as intracellular signals, which are lost in \textit{in vitro} cell culture models, can influence mitochondrial biology. Although nematodes lack many of the well-defined organs found in mammalian models, they do exhibit many distinct tissues such as the hypodermis, cuticle, gonad, intestine, muscles, and neurons. Elegant studies in nematodes have demonstrated that tissue-specific mitochondrial dysfunction can give rise to signaling events that alter mitochondrial function in other, distant tissues (120-123), thus demonstrating the importance of investigating mitochondrial function \textit{in vivo}.

1.9 \textit{Dissertation objectives and outline}

The primary objective to this work is to investigate if genetic deficiencies in mitochondrial fission, fusion, and mitophagy, or reduced mtDNA content sensitize the model organism \textit{C. elegans} to known and suspected mitochondrial toxicants. Given the complex nature of mitochondrial disease, I hypothesize that the aforementioned mitochondrial deficiencies will sensitize nematodes to some, but not all environmental toxicants.

In chapter 2, we optimize the Seahorse XF\textsuperscript{24} Extracellular Flux Analyzer for use with live \textit{C. elegans}. Using this protocol we assessed the fundamental parameters of mitochondrial respiration (basal oxygen consumption rate (OCR), maximal OCR, spare capacity, proton leak, and ATP-linked respiration) in fission-, fusion-, and mitophagy-deficient nematodes.
In chapter 3 we screen ten known and suspected environmental mitochondrial toxicants for exacerbation of larval growth delay in fission-, fusion-, and mitophagy-deficient nematodes. The mechanisms underpinning arsenite toxicity in fission- and fusion-deficient nematodes are then further investigated.

In chapter 4 we develop a *C. elegans* model of mtDNA depletion using a chronic, low-dose ethidium bromide exposure, and test five environmental toxicants for exacerbation of mitochondrial function in nematodes with reduced mtDNA content.

Chapter 5 is a summary of the results, and discusses some of the potential broader impacts of this work.
Figure 1: Process of fission, fusion, and mitophagy.

Figure adapted from (124).
2. Mitochondrial morphology and fundamental parameters of the mitochondrial respiratory chain are altered in Caenorhabditis elegans strains deficient in mitochondrial dynamics and homeostasis processes.

This bulk of this chapter was published under the same title in *PLoS One* in 2015 (PMID: 26106885). The authors are Anthony L. Luz, John P. Rooney, Laura L. Kubik, Claudia P. Gonzalez, Dong Hoon Song, and Joel N. Meyer. The fundamental parameters of the mitochondrial respiratory chain were analyzed for fourth larval stage fusion (*eat-3 (ad426))-deficient nematodes, and the data, which was not included in the original publication, is included in this chapter.

2.1 Introduction

Mitochondria play many important roles in cellular and organismal health including apoptosis (125), retrograde signaling (126), Ca$^{2+}$ signaling (127), and the Krebs cycle (128); however, mitochondria are best known for ATP production via oxidative phosphorylation (OXPHOS). The importance of mitochondria in organismal health is highlighted by the fact that mitochondrial dysfunction is causal in myriad human diseases affecting at least 1 in 5,000 individuals (129), and has been implicated in contributing to many others (130, 131). Furthermore, many drugs (132) and pollutants (66) cause mitochondrial dysfunction, in some cases only in specific (sensitive) genetic backgrounds (90, 91), thus emphasizing the need for a better understanding of the
effects of genetic deficiencies and environmental exposures on mitochondrial health in vivo.

Mitochondria are dynamic organelles that respond to cellular and/or environmental cues through fission and fusion. These interlinked processes are critical for maintaining proper mitochondrial function, number and shape (35). Mitochondrial dynamics are also crucial for stress response, as damaged mitochondria can fuse, allowing contents to mix, and then undergo fission generating two healthy mitochondria in a process termed functional complementation (133). Damaged mitochondria can also undergo fission, segregating damaged components, which can undergo degradation via targeted autophagy or mitophagy (134), thus preserving a healthy mitochondrial network. Further highlighting the importance of mitochondrial dynamics in organismal health, mutations in human fusion genes OPA1 and MFN2 cause dominant optic atrophy (43) and Charcot Marie Tooth Neuropathy type 2A (44), respectively, while mutations in mitophagy genes PINK1 and PARK2 cause familial Parkinson’s disease (42), and mutations in the fission gene, DRP1, have been associated with rare cases of neurodegeneration and early death (46).

Experiments in whole organisms are important because intercellular signals and cellular context that may affect mitochondrial function can be lost in in vitro experiments (135). Caenorhabditis elegans is a free-living nematode found largely in decaying leaf litter (136). As a model organism C. elegans offers many advantages over traditional
mammalian models, including a short (2-3 week) lifecycle, ease of maintenance, and potential for medium-throughput experiments (137, 138). Conservation of many molecular and cellular pathways (139), a fully sequenced and annotated genome (140), availability of genetic mutants (141), and ease of genetic knockdown via RNA interference (142, 143) contribute further to the utility of *C. elegans* as a model for studying mitochondrial dysfunction *in vivo*. Tools for assessment of mitochondrial function in *C. elegans* currently include time-consuming biochemical analysis of extracts (144), *in vivo* analysis of ATP levels using a transgenic reporter (145), and analysis of oxygen consumption (basal respiration) using individual or multi-well plate formats (146).

Here we describe how to assay the fundamental parameters of mitochondrial respiratory chain function, including basal oxygen consumption rate (OCR), maximal respiratory capacity, spare respiratory capacity, ATP coupled respiration, and proton leak with pharmacological inhibitors of the electron transport chain (ETC) in the model organism *C. elegans* using the Seahorse XF-24 Analyzer (Seahorse Bioscience, Massachusetts, USA). Furthermore, we report alterations in these parameters in nematodes carrying mutations in orthologs of the human outer membrane fusion gene *MFN2*, inner membrane fusion gene *OPA1*, mitochondrial fission gene *DRP1*, mitophagy genes *PINK1* and *PARK2*, and a complex III Rieske iron sulfur protein (*fzo1, eat-3, drp-1, pink-1, pdr-1* and *isp-1*, respectively, in *C. elegans*). These results highlight the importance
of mitochondrial dynamics in maintaining proper mitochondrial function. Clearly, however, the analysis of mitochondrial function in nematodes carrying mutations in genes of other critical mitochondrial pathways, such as apoptosis, the ETC, the Krebs cycle or fatty acid oxidation will help us better understand connections between these pathways and mitochondrial energetics.

2.2 Methods

2.2.1 C. elegans culture conditions

Bristol N2 (wild-type), MQ887 isp-1 (qm150; outcrossed 3x), VC1024 pdr-1 (gk448; outcrossed 3x), and CB6193 bus-8 (e2885; outcrossed 3x) C. elegans were purchased from the Caenorhabditis Genetics Center (CGC, University of Minnesota). CU5991 fzo-1 (tm1133; outcrossed 4x) and eat-3 (ad426; outcrossed >1x) were provided by Alexander van der Bliek, University of California (Los Angeles, CA, USA), pink-1 (tm1779; outcrossed 1x) were provided by Guy Caldwell, University of Alabama, and CU6372 drp-1 (tm1108; outcrossed 9x) were provided by Ding Xue, University of Colorado. All mutant strains will henceforth be referred to by their gene name. Synchronized populations of C. elegans were obtained by sodium hydroxide bleach treatment as previously described (147), followed by overnight incubation in complete K-medium on a shaker at 20C (148). Age synchronized L1 (larval stage one) nematodes were then maintained at 20C on K-agar plates (149) seeded with OP50 Escherichia coli until L4
(larval stage four) was reached (approximately 48 hours for N2, *drp-1, pdr-1, pink-1* and 72 or 96 hours for slow growing *fzo-1* and *isp-1*, respectively).

### 2.2.2 Drug preparation

Dicyclohexylcarbodiimide (DCCD), oligomycin A, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 2,4-dinitrophenol (Sigma Chemical Co., St. Louis, MO) stocks were prepared in dimethyl sulfoxide (DMSO), and diluted in unbuffered reconstituted hard water (“EPA water” hereafter) (60mg MgSO$_4$·7H$_2$O, 60mg CaSO$_4$·2H$_2$O, 4mg KCl per liter ddH$_2$O) (150) to their final working concentrations. Sodium azide (Sigma Chemical Co., St. Louis, MO) was dissolved in unbuffered EPA water to a final working stock of 80mM.

### 2.2.3 Sample preparation

All experiments were performed with synchronized L4 *C. elegans*, as the L3/L4 transition is accompanied by a dramatic increase in mtDNA copy number and demand for oxidative phosphorylation (112, 151-153). L4 *C. elegans* were rinsed from OP50 K-agar plates into sterile 15mL centrifuge tubes, washed twice with K-medium, and allowed to clear their guts for 20 minutes to remove contaminating bacteria that might otherwise confound oxygen consumption rate (OCR) measurements. Next, nematodes were resuspended in unbuffered EPA water to an approximate concentration of one worm per microliter (estimated by counting the number of worms in 20µl drops). Approximately 75 nematodes were then pipetted into each well of a 24-well Seahorse
utility plate using tips rinsed in 0.1% Triton X-100 to prevent worm loss due to sticking. The final volume of each well was then brought to 525µl with unbuffered EPA water. At least two wells per assay were left as blanks. 75µL of 160µM DCCD (8% DMSO), 120µM FCCP (16% DMSO), and 80mM azide were then pipetted into the appropriate injection ports of the seahorse cartridge. After injection, each drug solution is diluted by a factor of eight to the appropriate final concentrations (i.e. 20µM DCCD (1% DMSO), 15µM FCCP (2% DMSO), and 10mM azide).

Seahorse programs were set up such that each oxygen consumption measurement consisted of a one minute mix cycle (which oxygenates the micro-chamber), followed by a three minute wait period (to allow worms to settle), and finally a three minute interval for measurement of oxygen levels. Eight oxygen consumption measurements were taken for determination of basal OCR. Drugs were then injected, and fourteen, eight or four OCR measurements were taken at eight minute intervals after DCCD, oligomycin, FCCP, or azide injections, respectively. Over the course of the assay it is important to monitor oxygen levels per well, as exposing the nematodes to hypoxic conditions could confound the measurements; oxygen levels below 100 mmHg were taken as an indicator of excess number of nematodes per well (Kevin Bittman, Ph.D., Field Applications Scientist, Seahorse Biosciences, Inc., personal communication). It is also critical to note whether the mix cycle is fully re-oxygenating the micro-chamber, and to readjust the mix cycle as needed.
Basal OCR measurements were highly variable over the initial four readings, but then stabilized. Therefore, we averaged the final four measurements to obtain an average basal OCR per well. The nematodes’ response to DCCD was not instantaneous, but OCR measurements consistently decreased, and plateaued between the sixth and eighth measurements. Therefore, we averaged the final six measurements to obtain our OCR in response to DCCD. C. elegans’ response to FCCP, although not instantaneous, was much more rapid than for DCCD. Thus, we averaged the final six measurements to obtain the average OCR in response to FCCP. Response to azide was essentially instantaneous, so we averaged all four measurements. Finally, we normalized all data to both worm number and total protein as measured by BCA assay (Thermo Fisher Scientific, Rockford, IL). Using this method we treated each individual well as an “n” of one. All experiments were run at least two to three times, separated in time.

Typically, when using a Seahorse instrument with cells in culture, inhibitors are injected into each well of a seahorse utility plate in tandem, such that after basal OCR is measured the ATP synthase inhibitor is injected (DCCD or oligomycin), followed by the mitochondrial uncoupler (2,4-DNP or FCCP), and finally by a complete respiratory inhibitor (sodium azide or rotenone plus antimycin A). This strategy allows for the determination of basal OCR, maximal respiratory capacity, spare respiratory capacity, proton leak, and ATP turnover for each well. However, this strategy does not appear to be possible in C. elegans. When we injected the cytochrome c oxidase inhibitor sodium
azide after the final FCCP measurement, we found that the magnitude of the nematode’s response to sodium azide was diminished (Figure 2, one way ANOVA, main effect of treatment P<.0001). To avoid this problem, sequential injection of other complete respiratory inhibitors such as cyanide or rotenone and antimycin A may be possible. In the experiments reported here, each drug was injected into a separate well to obtain reliable, reproducible results.

2.2.4 Extracellular acidification rate (ECAR)

Due to the dual probe capacity of the Seahorse XF24 it is possible to obtain oxygen consumption and extracellular acidification rate (ECAR) simultaneously, which is why all assays were run in unbuffered EPA water. Simultaneous measurements of OCR and ECAR have proven valuable in the context of toxicant exposures that can cause a shift in metabolism from OXPHOS to aerobic glycolysis (154), otherwise known as the Warburg effect (155). To test whether we could measure ECAR in C. elegans, we tested ECAR in wild-type and fzo-1 nematodes. fzo-1 (tm1133) nematodes, in which mitochondria are highly fragmented and exhibit intracellular acidification, likely due to increased glycolysis, because sodium dichloracetate (a pyruvate dehydrogenase stimulator) alleviates acidosis (156). However, no differences in ECAR were noted between L4 stage N2 and fzo-1 nematodes (Figure 3, one-way ANOVA, P>0.05). We speculate that extrusion of glycolytic byproducts in C. elegans (such as lactate) does not
occur in the same manner as cells in culture, thus limiting the value of ECAR measurements.

2.2.5 Mitochondrial morphology

*C. elegans* were picked onto agar plates seeded with either OP50 (negative control) or OP50 containing 3.7µM MitoTracker Red CMXROS (Molecular Probes, Invitrogen) and incubated overnight. Worms incubated with MitoTracker were picked onto plates seeded with OP50 for 30 minutes the next day to allow the dye to clear out of the gut. Each strain was then picked onto an agar pad containing levamisole (25mg/mL) and subsequently imaged on a confocal microscope (Zeiss 510 upright, Duke Light Microscopy Core Facility).

Raw images were converted to binary images using MATLAB. First, maximum projection technique as described in (157) was applied in order to combine z-stack images into a single image. Gray values of this image were linearly transformed to cover the entire 16 bit gray scale. These images were then deblurred using the ‘deconvblind’ function. The experimentally measured point spread function (PSF) was applied as an initial PSF for this deconvolution process. A Gaussian low pass filter was applied to smooth the edges of the boundary. Finally, these images were converted to the binary images using Otsu’s method (158).

As can be seen in Figure 4, the centerline of a mitochondrion was extracted from the binary image using a morphological thinning operation in MATLAB (‘bwmorph’
function with ‘thin’ operation). After the thinning operation, the endpoints of the centerline were connected to the boundary of a mitochondrion to complete the centerline. The average width of a mitochondrion was then calculated as area divided by the centerline length. The aspect ratio (AR) was defined as the ratio between centerline length and average width (multiplying by $\pi/4$ allows the aspect ratio of a circle to be one).

$$\text{AR} = \frac{\pi \text{ centerline length}}{4 \text{ average width}}$$

As we observed some nonspecific dye uptake in the gut, we measured mitochondria from lateral body wall muscle cells from three worms within a strain (n=108-173). Our analysis focused on strain-to-strain differences and pair wise comparisons to wild type nematodes were performed for each strain using a one way ANOVA.

### 2.2.6 Statistics

All statistics were performed using JMP v11.0 software (SAS Institute). All OCR data was initially assessed with a one or two way ANOVA. When appropriate, post-hoc analysis was carried out using a Student’s t-test.
2.3 Results

2.3.1 Drug Titrations

We first carried out titrations of inhibitors of various ETC components to allow us to measure different aspects of mitochondrial function.

*Oligomycin & DCCD.* Oligomycin and DCCD are ATP synthase inhibitors that bind the F\textsubscript{0} and F\textsubscript{0}F\textsubscript{1} subunits of the complex, respectively, preventing proton translocation and phosphorylation of ADP to ATP (159). Inhibition of ATP synthase provides a measure of the amount of oxygen consumption coupled directly to ATP production. However, in N2 nematodes, oligomycin proved ineffective at inhibiting ATP synthase at all concentrations tested (5-50µM, 2% DMSO), likely due to limited penetration of the nematode’s collagenous cuticle by this bulky compound (160) over the timeframe of the assay (up to 15 cycles tested, or roughly 1.75 hours) (Figure 5). Higher concentrations of oligomycin could not be tested, due to its limited water solubility. Citreoviridin A, another ATP synthase inhibitor (161, 162), was also tested, but to no effect.

DCCD inhibited ATP synthase more effectively than either oligomycin or citreoviridin. DCCD had a significant effect on OCR (one-way ANOVA, main effect of treatment P<0.0001). 10 and 20µM DCCD significantly reduced OCR from basal levels (P<0.0001 for both pairwise comparisons), while 5 and 50µM DCCD did not alter OCR (P=0.2 and 0.3, respectively, for pairwise comparisons). DCCD is a water insoluble
compound and it is plausible that DCCD precipitated out of solution over the time course of the assay, explaining why 50µM DCCD failed to effect OCR. However, our 2% DMSO control caused a slight but significant increase in OCR rates (P=0.004 for pairwise comparison) (Figure 6). To avoid this confounding DMSO effect, we reduced our final DMSO concentrations to 1%, the lowest possible DMSO concentration where 20µM DCCD, the most effective concentration tested, was soluble. DCCD significantly reduced OCR at all concentrations of DMSO tested (one-way ANOVA, main effect of treatment, P<0.0001). 20µM DCCD at 1, 1.5, and 2% final DMSO concentrations all significantly reduced OCR (P<0.05 for all pairwise comparisons), while DMSO concentration did not affect the efficacy of 20µM DCCD in reducing OCR (P>0.05 for all pairwise comparisons) (Figure 7). Since 1% DMSO did not significantly affect basal OCR (P=0.5 for pairwise comparison), we chose to use 20µM DCCD at a final DMSO concentration of 1% for all future experiments. Representative Seahorse XF-24 output data for 20µM DCCD (1% DMSO) is shown in Figure 8A.

Although DCCD and oligomycin are both ATP synthase inhibitors, DCCD lacks the specificity of oligomycin and is capable of inhibiting other cellular ATPases; thus, it gives an imperfect measure of ATP-linked respiration and proton leak (159). We explored two alternatives that might permit the use of oligomycin instead of DCCD. First, we attempted long-term (12H) pre-incubation with oligomycin and second, we titrated DCCD and oligomycin in cuticle-deficient bus-8 nematodes that have also been
reported to be hypersensitive to several bulky drugs (163). A 12 hour pre-incubation with oligomycin resulted in a significant reduction in basal OCR in *bus-8* nematodes (one-way ANOVA, main effect of treatment P<0.0001). 10, 25 and 50µM oligomycin significantly reduced basal OCR (P=0.004, P<0.0001, P<0.0001, respectively, for all pairwise comparisons), with 50µM causing roughly a 40% reduction in OCR (Figure 9A). When injected in real-time, both oligomycin and DCCD inhibited ATP synthase in *bus-8* nematodes and caused significant reductions in OCR (one-way ANOVA, main effect of treatment P=0.0007 and P<0.0001, respectively). 25 and 50µM oligomycin caused significant reductions in OCR (P=0.0017 and P=0.005, respectively, for pairwise comparisons), whereas 10µM had no effect (P>0.05) (Figure 9B). 5, 10 and 20µM DCCD caused significant reductions in OCR (P<0.0001 for all pairwise comparisons) and 1µM had no effect (P>0.05) (Figure 10). The magnitude of the effect for the two drugs were significantly different when we compared the percent reduction in basal OCR after administration of 20µM DCCD or 50µM oligomycin (one-way ANOVA, P=0.0005) (Figure 11), with DCCD causing roughly a 55-60% decrease and oligomycin causing a 35-40% decrease.

**FCCP & 2,4-Dinitrophenol.** FCCP and DNP are potent uncouplers of mitochondrial oxidative phosphorylation (164, 165) that dissipate the proton gradient in the mitochondrial intermembrane space by transporting protons across the inner mitochondrial membrane independently of ATP synthase activity. Once in the
mitochondrial matrix, uncouplers deprotonate, cross back into the intermembrane space, and repeat the cycle, thus uncoupling oxygen consumption from ATP production (oxygen is still consumed to generate and maintain the proton gradient, but ATP is not produced). Dissipation of the proton gradient forces increased fuel oxidation, oxygen consumption, and thus proton pumping in an attempt to re-establish the proton gradient. Therefore, uncoupling provides a useful measure of maximal respiratory capacity, or an organism’s ability to respond to increasing energy demands (166).

2,4-DNP (25-100µM, 2% DMSO) had no effect on mitochondrial respiration over the time course tested (Figure 12), while a near instantaneous increase in OCR was observed after FCCP treatment. Other groups have also reported a lack of mitochondrial uncoupling after 2,4-DNP treatment in nematodes (167-169); however the exact reason for this is unknown. One possible explanation for the lack of effect of 2,4-DNP is that it is highly ionized (776:1, based on a pKₐ of 4.11) when injected in EPA water (pH~7), whereas FCCP is less so (16:1, based on a pKₐ of 5.8), and it is likely the ionized compounds do not penetrate the nematode cuticle efficiently. Thus titration experiments were performed to identify the appropriate FCCP concentration for effective mitochondrial uncoupling.

FCCP treatment had a significant effect on OCR (one-way ANOVA, P<0.0001). 5, 15, 25 and 50µM FCCP (2% DMSO) significantly increased OCR measurements above basal rates (P<0.0001 for all pairwise comparisons to control), while our 2% DMSO
control did not significantly increase OCR (P=0.3 for pairwise comparison) (Figure 13). The fact that our 2% DMSO control failed to significantly increase OCR in our FCCP trials despite increasing OCR after DCCD injections is likely due to the different lengths of the assays, as we measure OCR 14 times post-DCCD injection, while only eight times post-FCCP. In contrast to DCCD, titrating our final DMSO concentrations downward resulted in increased variability in the FCCP response (Figure 14) so we chose to run all future experiments with 2% DMSO. Since 15, 25, and 50µM FCCP did not elicit statistically significantly different responses (P>0.05 for all pairwise comparisons), we ran all future experiments with the lowest concentration of FCCP, 15µM, which is similar to concentrations other groups have used to uncouple respiration in nematodes (170). Representative Seahorse XF24 output data for 15µM FCCP (2% DMSO) is shown in Figure 8B.

**Sodium Azide.** Azide is a powerful inhibitor of mitochondrial respiration, and works by inhibiting complex IV (cytochrome c oxidase (COX)) of the ETC by binding directly to the heme prosthetic group, preventing the final transfer of electrons to oxygen (171, 172). The selectivity of azide as a COX inhibitor has been questioned, as early *in vitro* studies suggested that azide promiscuously inhibits myriad heme containing enzymes. However, azide binding is pH dependent due to its pKa (4.7), and at physiological pHs azide preferentially exists as the free anion (N₃⁻). Most heme containing enzymes preferentially bind the protonated form of azide, but COX is an
exception, binding the anion with greater affinity (173). This suggests that azide has high specificity for COX inhibition \textit{in vivo}, making it an ideal drug for our studies.

Treatment with sodium azide had a significant effect on nematode OCR (one-way ANOVA, P<0.0001). 2.5, 5, 10, and 15mM azide all significantly reduced OCR below basal levels (P<0.0001 for all pairwise comparisons to control), and the azide response was not statistically different for any of the azide concentrations tested (P>0.05 for all pairwise comparisons) (Figure 15). All future experiments were run with a final concentration of 10mM. Representative Seahorse XF\textsuperscript{24} output data for 10mM azide is shown in Figure 8C.

\textbf{2.3.2 Mutant \textit{C. elegans} metabolic profiles and mitochondrial morphologies}

Having identified appropriate conditions for analysis of mitochondrial function, we next carried out analyses of mitochondrial function in strains of \textit{C. elegans} carrying mutations in critical mitochondrial function and homeostasis genes. Because some of the strains we used grow to different sizes, we report our results on a per unit protein basis. Alterations in mitochondrial morphology for many, but not all of these mutants have been previously reported; we carried out further morphological analysis using a dye-based imaging technique and novel imaging method that complements previous literature. All results are summarized in Table 1.
**Basal OCR.** First, we investigated whether *C. elegans* deficient in mitochondrial fission (*drp-1*), fusion (*fzo-1, eat-3*), mitophagy (*pdr-1, pink-1*) or ETC complex III (*isp-1*) activity have altered basal oxygen consumption. Since mitochondrial dynamics are critical in maintaining healthy mitochondrial networks (35), we hypothesized that disruption of these genes would result in altered patterns of oxygen consumption.

Basal oxygen consumption was measured using 75 L4 N2, *drp-1, pdr-1* and *pink-1* nematodes. 150 L4 *fzo-1, eat-3*, and *isp-1* nematodes were required to meet the Seahorse XF24’s range of detection (40-1400 pmols/min), presumably because L4 *fzo-1, eat-3* and *isp-1* are smaller than wild-type nematodes. Basal oxygen consumption per unit protein was significantly different between strains (one way ANOVA, main effect of strain, P<.0001) (Figure 16, Figure 17). *drp-1* nematodes had significantly elevated oxygen consumption compared to wild-type (N2) nematodes (P=.0006 for pairwise comparison), which is in agreement with the idea that highly fused mitochondrial networks are more metabolically active (174), while *eat-3* nematodes had reduced oxygen consumption (P=0.0005). Interestingly, we did not observe decreased oxygen consumption in *fzo-1* or *isp-1* nematodes, which have a missense mutation in the Rieske iron sulfur protein subunit of complex III.

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1 Seahorse XF analysis of *eat-3* (Figure 17) was not included in the original publication, but was measured at a later time alongside wild-type nematodes, which were used for all comparisons.
**ATP coupled respiration.** Inhibition of ATP synthase by DCCD provides a measure of the amount of oxygen consumption coupled to ATP production. We hypothesized that mutations in mitochondrial dynamics genes and/or complex III of the ETC, which induce mitochondrial dysfunction, would alter this parameter.

Treatment of N2, *drp-1*, *pdr-1*, *pink-1*, *fzo-1*, and *isp-1* with 20µM DCCD caused a significant reduction in OCR in all strains (Figure 18) (two-way ANOVA, main effect of strain and treatment, P<0.0001 for both, but not their interaction, P=0.65); however, ATP coupled respiration was only reduced in *eat-3* nematodes (Figure 17C) (P=0.0013).

**Maximal respiratory and spare respiratory capacity.** Uncoupling ATP production from oxygen consumption with FCCP provides a measure of maximal respiratory capacity. When basal OCR is subtracted from maximal OCR, the result is spare respiratory capacity, an important measure of an organism’s ability to respond to increasing energy demands. Treatment of N2, *drp-1*, *pdr-1*, *pink-1*, *fzo-1*, and *isp-1* with 15µM FCCP caused a significant increase in OCR above basal levels in all strains (two-way ANOVA, main effects of strain (P<.0001), treatment (P<.0001) and their interaction (P<.0001)) (Figure 19A, Figure 17D). *isp-1*, *fzo-1*, *eat-3*, and *drp-1* had significantly reduced FCCP responses compared to wild-type nematodes (P=0.03, P<0.0001, P<0.0001, P=0.01, respectively, for pairwise comparisons) (Figure 17, Figure 19).

Strain-specific differences were observed when spare respiratory capacity was compared (one way ANOVA for effect of strain, p=0.022). Post-hoc analysis showed that
drp-1, fzo-1, and eat-3 have reduced spare respiratory capacities compared to wild-type (N2) nematodes (P=0.013, P=0.02, P=0.0014 respectively, for pairwise comparisons) (Figure 19B, Figure 17E). 2% DMSO controls were run in conjunction with FCCP for each strain; however, DMSO had no significant effect on OCR when normalized to total protein (Figure 20, two way ANOVA, P>0.05).

Proton Leak. Proton leak is defined as dissipation of the proton gradient across the inner mitochondrial membrane that is not attributable to ATP synthase activity. Basal proton leak mainly occurs via the adenine nucleotide translocase (ANT), which is not regulated, but can differ between cell types (175). Inducible proton leak is regulated, and is mainly mediated by uncoupling proteins (UCP), which are inducible by fatty acids, superoxide, and by-products of lipid peroxidation (175). The exact role of inducible proton leak outside of generation of heat is not fully understood; however, one hypothesis is that proton leak serves a role in a negative feedback loop to limit superoxide production (18). Mitochondrial dysfunction, caused by mutations in mitochondrial dynamics and ETC complex III, might result in altered levels of proton leak either directly or indirectly, e.g. via compensatory responses.

Basal OCR was significantly depressed upon injection of sodium azide in all strains tested (main effect of drug (P<.0001), but not strain (p=0.15) or their interaction (p=0.60)) (Figure 21). Injection of DCCD and sodium azide caused significantly different reductions in OCR (two-way ANOVA, main effects of strain (P<0.0001), treatment
(P<0.0001) and their interaction (P=0.0002)) in N2, *pdr-1*, *pink-1* and *drp-1*, but not in *fzo-1* or *isp-1* (P=0.017, P<0.0001, P<0.001, P<0.0001, P=0.43, P=0.96, respectively, for all pairwise comparisons) (Figure 22A). Comparing proton leak proved more challenging in nematodes than in cell culture, as we did not inject our ATP synthase inhibitor (DCCD) and complete respiratory inhibitor (sodium azide) into the same wells. Therefore, because we did not have matched samples to compare, we subtracted all azide response OCR measurements from the average DCCD measurement for each strain. Using this approach, we observed dramatic and significant strain differences (one way ANOVA, P<0.0001). Interestingly, *isp-1* and *fzo-1* nematodes had reduced proton leak compared to wild-type nematodes, while *pink-1* had significantly elevated proton leak (P=0.005, P=0.04, P=0.004, respectively, for pairwise comparisons to control) (Figure 22B).

**Mitochondrial morphology.** As mitochondrial morphology can play a role in metabolic activity, we imaged muscle cell mitochondria and quantified the mitochondrial aspect ratio for each strain. The aspect ratio serves as a proxy for measuring the extent to which mitochondria are networked; a higher aspect ratio indicates more highly fused mitochondria (see Methods for more details). Results are summarized in Table 1. And representative images are shown in Figure 23. As expected, *fzo-1*-deficient nematodes had a significantly reduced aspect ratio compared to wild-type *C. elegans* (one way ANOVA, main effect of strain P<0.0001). Interestingly, both *pdr-1* and *pink-1* had significantly larger aspect ratios compared to N2 (one way ANOVA,
main effect of strain P=0.032 and P=0.016, respectively). Surprisingly, \textit{drp-1}-deficient nematode’s aspect ratio was not statistically different from wild-type \textit{C. elegans} (one way ANOVA, P>0.05). Because this result was unexpected, \textit{drp-1} nematodes were genotyped alongside N2 \textit{C. elegans} and the 425bp deletion was confirmed. Although \textit{isp-1}-deficient nematodes have been previously reported to have fragmented mitochondrial networks (176), we could not confirm this, as uptake of MitoTracker Red CMXROS dye was poor, likely due to \textit{isp-1} nematode’s reduced mitochondrial membrane potential (177, 178).

\section*{2.4 Discussion}

Here we present a method to assess the fundamental parameters of mitochondrial function \textit{in vivo}, using the model organism \textit{C. elegans}: basal and maximal OCR, spare respiratory capacity, ATP-coupled respiration, and proton leak. Furthermore, we identify strain-specific differences in these parameters in mitochondrial fission (\textit{drp-1}), fusion (\textit{fzo-1, eat-3}), mitophagy (\textit{pdr-1 & pink-1}), and ETC complex III (\textit{isp-1}) deficient nematodes.

Although measuring mitochondrial function \textit{in vivo} offers many advantages compared to \textit{in vitro} assays, the method we describe here does have limitations. For example, the inability to inject drugs sequentially into each well reduces the throughput of the assay; however, this could potentially be overcome by adapting our protocol to the Seahorse XF-96 Analyzer. Furthermore, the dual probe nature of the XF-24 Analyzer allows for the simultaneous measure of both OCR and ECAR; however, ECAR
measurements appear to have limited utility in C. elegans. Additionally, the Seahorse XF²4 Analyzer lacks cooling capacity, and tends to warm above room temperature as the assay progresses (on average to 25°C). Thus, temperature may be a concern in longer assays. Storage of the XF²4 Analyzer in a temperature and humidity controlled enclosure may solve this issue.

Another limitation of this assay is the lack of a highly specific ATP synthase inhibitor that works in nematodes. DCCD, an Fo:F₁ inhibitor, also inhibits other ATPases that can contribute to oxygen consumption (159), making ATP-linked respiration and proton leak measurements difficult to interpret. Unfortunately, the highly specific Fo inhibitor oligomycin appears to work only after very long incubation periods or in cuticle-deficient bus-8 nematodes, limiting its practicality. Nevertheless, oligomycin could be used in conjunction with RNAi in a bus-8-deficient background to study the interactions between genetic knockdown, toxicant exposure and mitochondrial dysfunction. However, oligomycin only caused a 36% reduction in OCR, suggesting that only 36% of oxygen consumption is coupled to ATP production. This is a significantly lower value than normally reported, and we suspect that oligomycin is not completely inhibiting ATP synthase, even in a cuticle deficient background. A lack of complete inhibition could still be due to poor penetration of the nematode by oligomycin, or incomplete organismal diffusion leaving ATP synthase active in certain cell types. The most effective concentration of DCCD caused roughly a 20% greater decrease in OCR.
than oligomycin (Figure 11); however, it seems unlikely that inhibition of nonspecific ATPases would contribute such a large percentage to OCR, further suggesting that oligomycin is not causing complete ATP synthase inhibition. We hypothesize that the 20% difference in DCCD and oligomycin responses are due to a combination of nonspecific inhibition of ATPases and incomplete inhibition of ATP synthase by oligomycin. Thus no matter which inhibitor is chosen for a study, results must be interpreted cautiously, and when possible confirmed via alternative methods, such as direct measurement of ATP levels.

Alterations in basal OCR were observed in drp-1 (increased) and eat-3 (decreased) nematodes. In agreement with this finding, highly fused mitochondrial networks, which have been observed in drp-1 (tm1108) knockout C. elegans (179), are often associated with increased metabolic activity (174), while fragmented mitochondria are often associated with decreased metabolic activity. Interestingly, we did not observe significantly different mitochondrial morphology between drp-1 and N2 nematodes in the current study. Instead, wild-type nematodes appeared to have hyper-fused mitochondrial networks, which we speculate is either life-stage or cell-type specific (i.e. muscle cells). For example, at the L3/L4 transition a dramatic increase in mtDNA copy number occurs as demand for oxidative phosphorylation increases (112, 151, 152). As highly fused mitochondria are typically more metabolically active (174) it is plausible that mitochondrial fusion also occurs in wild-type nematodes at the L3/L4 transition to help
meet rising energy demands. However, it is important to note that mutations in DRP1 are associated with human disease (46), and mitochondrial dysfunction has been reported in drp-1 (tm1108) nematodes and Purkinje cells of DRP1 deficient mice (180). Previously, we reported reduced ATP levels and an increased mtDNA:nDNA ratio in L4 drp-1 nematodes (76), while others have reported decreased brood size (179).

Surprisingly, neither fzo-1 nor isp-1 nematodes had altered basal OCR, despite the fact that we (Figure 16) and others have observed fragmented mitochondrial networks in fzo-1 and isp-1 (156, 176, 181). Reductions in basal OCR have been reported in fzo-1 nematodes on a per worm basis (182), and mild reductions in L1 and mixed populations (normalized to either total protein or body volume) of isp-1 nematodes (183, 184) have also been reported, neither of which were observed in our age-synchronized L4 populations. A limitation of these normalization methods is that they do not take into account potential strain specific differences in mitochondrial mass. The use of transgenic reporter strains expressing mitochondrial localized green fluorescent protein to estimate mitochondrial mass may improve normalization. However, similarly to drp-1 deficient C. elegans, isp-1 nematodes have also been reported to have reduced ATP levels (184, 185), thus highlighting the necessity of measuring multiple endpoints in assessing mitochondrial health.

The fact that mutations in mitochondrial dynamics and complex III genes did not cause stronger phenotypes was surprising. One possibility is that this is the result of
compensatory mechanisms, which have indeed been reported in ETC and mitochondrial
dynamics mutant nematodes. For example, *fzo-1* nematodes appear to upregulate
glycolysis in an attempt to maintain energy homeostasis (156), while *MFN1/MFN2-
deficient skeletal myocytes and Purkinje cells attempt, but fail, to maintain energy
homeostasis by increasing mitochondrial biogenesis (186, 187). Likewise, complex I (*gas-
1*)-deficient nematodes have compensated for decreased complex I activity by
upregulating transcription of other OXPHOS genes (in particular complex II and III), as
well as genes involved in the TCA cycle, glycolysis and fatty acid metabolism (188).
Interestingly, complex III mutants (*isp-1*) appear to downregulate transcription of
complex I, perhaps in an attempt to limit ETC-derived reactive oxygen species (ROS)
(188). Of course other, as yet unidentified, compensatory mechanisms are likely also
playing a role, such that it is not surprising that major mitochondrial dysfunction was
not detected until chemical challenge.

Exposure to the mitochondrial uncoupler FCCP revealed reduced maximal
respiratory capacities in *isp-1, fzo-1, eat-3*, and *drp-1* nematodes, while only *drp-1, fzo-1,
eat-3* had significantly reduced spare respiratory capacities. These results are somewhat
surprising, as highly fused mitochondrial networks are often associated with increased
metabolic activity (174). However, mitochondrial homeostasis is maintained through the
interlinked processes of fission and fusion, and a lack of either is associated with
mitochondrial dysfunction in humans (44) and *C. elegans* (156). Despite their shared loss
in spare respiratory capacity, we have previously shown that *drp-1* nematodes are mildly resistant to UVC induced mtDNA damage, while *fzo-1* and *eat-3* *C. elegans* are hypersensitive (76, 148). These differences are likely due to the buffering effect of increased fusion in *drp-1*, which has been lost in *fzo-1* and *eat-3* nematodes. Nonetheless, these findings further highlight the importance of fission and fusion in maintaining proper mitochondrial function.

Interestingly, we observed increased proton leak in *pink-1*, and decreased leak in *fzo-1* and *isp-1* nematodes. Increased levels of ROS, the principle known inducer of uncoupling protein 2 (UCP2), which typically mediates inducible proton leak (18, 175), have been measured in *pink-1* deficient cell lines (189) and in mitochondria isolated from Parkinson’s disease patients (189-191). In agreement with our findings, increased ROS production, decreased ATP levels and decreased membrane potential, suggestive of proton leak, were recently reported in *pink-1*-deficient nematodes (192). However, the authors also measured increased basal OCR, and images of abnormal mitochondrial networks appeared fragmented, conflicting with our results (192). Increased ROS production in *pink-1*-deficient nematodes could induce uncoupling activity, resulting in mild uncoupling, reduced ATP levels (76), and increased proton leak, which would limit further ETC-derived ROS. Uncoupling activity is not well understood in nematodes and the sole uncoupling protein homolog, a UCP4 homolog, does not appear to have uncoupling activity, but is instead a succinate transporter (193). Interestingly, proton
leak did not differ from wild-type levels in pdr-1-deficient nematodes, although pdr-1-deficient nematodes have recently been reported to have reduced ATP levels, reduced mitochondrial membrane potential, and increased basal OCR, suggestive of proton leak (192). While pink-1 and pdr-1 both participate in the mitophagy pathway, different levels of proton leak in these two strains may be explained by the fact that pink-1 is a kinase that has many functions in addition to mitophagy, including regulation of mitochondrial respiration and ROS production (48, 194). Furthermore, emerging evidence suggests that cytosolic pink-1 can promote cell survival and neuron differentiation (195-197). Although proton leak differed between pink-1 and pdr-1, we report here for the first time that both strains have mitochondrial networks that are highly fused, exhibiting significantly larger aspect ratios compared to wild-type nematodes, which is consistent with the strains’ shared loss of mitophagy, and thus reduced mitochondrial turnover.

Increased production of superoxide has been measured in isp-1 nematodes by several groups (22, 198), which at first seems to conflict with our finding of decreased proton leak. However, superoxide appears to play both a beneficial and critical role in isp-1’s long lived phenotype, as supplementation with ROS scavengers reduced the long-lived phenotype of isp-1 (198). Instead of by increasing proton leak, isp-1 nematodes may limit excessive ROS production by downregulating OXPHOS, including one of the main sites of ROS production, complex I (188). Due to diminished complex I and III activity (188), it is likely that isp-1 nematodes struggle to generate a proton gradient and proton
leak would further limit ATP production in an organism that already has reduced ATP levels (184). In agreement with this, several groups have reported reduced mitochondrial membrane potential in *isp-1*-deficient nematodes (177, 178), which also explains the lack of uptake of MitoTracker Red CMXROS dye, and our inability to quantify mitochondrial morphology in *isp-1*-deficient nematodes.

Mitochondrial function appears to deviate most from wild-type in fusion (*fzo-1, eat-3*)-deficient nematodes, which have reduced basal OCR (*eat-3* only), maximal respiratory capacity, spare respiratory capacity, proton leak (*fzo-1* only), and ATP-coupled oxygen consumption (*eat-3* only). These findings are not surprising as impaired respiration has been reported in *MFN1/MFN2* deficient cardiac myocytes (199), skeletal myocytes (187), and in Purkinje cells (186). In accord with mitochondrial dysfunction, *fzo-1* and *eat-3* nematodes have highly fragmented mitochondria and develop slowly; however, their AMP/ATP ratio does not appear altered, suggesting that loss of *fzo-1* does not significantly alter energy production (156). Instead, *fzo-1*-deficient nematodes may upregulate glycolysis in order to maintain energy homeostasis (156) and/or alter behavior to reduce ATP use. In agreement with our findings, decreased proton leak has been reported in 10T1/2 cells transfected with antisense mouse *MFN2* (200); however, this group also reported reductions in basal OCR. Interestingly, upregulation of *ANT3*, but not the ROS inducible *UCP2*, has been observed in mitochondria isolated from CMT2A patients (201), suggestive of increased proton leak, but not superoxide
production, which conflicts with our findings. However, to our knowledge, mitochondrial uncoupling has not been investigated in \textit{fzo-1} nematodes, and as superoxide production does not exceed wild-type levels, mitochondrial uncoupling would not be expected to be upregulated via ROS production (182). Since proton leak would further reduce OXPHOS derived ATP production by dissipating the proton gradient, it is likely that proton leak would not be advantageous to \textit{fzo-1} nematodes.

Reports of ATP-coupled respiration vary in \textit{in vitro} studies of MFN2-deficiency. Mitochondria in fibroblasts isolated from CMT2A patients have been reported to have decreased coupling (201, 202), while others have reported no effect (37, 200). Interestingly, \textit{MFN2} knockout L6E9 myotubes appears to repress OXPHOS, as nuclear encoded subunits of complex I, II, III, and V are all downregulated, while glucose transport, and lactate production were upregulated, suggesting a compensatory switch to glycolysis to maintain ATP levels (37). On the other hand, fibroblasts from CMT2A patients, which have reduced coupling efficiency, maintained ATP levels by increasing oxygen consumption and complex II activity (201). Although not significant, we did observe a trend toward increased ATP-coupled respiration in \textit{fzo-1} nematodes (one way ANOVA, \( P=0.07 \), S11B Fig.), which is in agreement with our finding of decreased proton leak, as leak tends to result in mild uncoupling. It is likely that many of these discrepancies are due to the complex nature of \textit{MFN2}, the role it plays in different cell
types, or the varying effects knockout versus knockdown versus mutations ultimately have on protein function.

2.5 Conclusions

We describe measurement of the fundamental parameters of mitochondrial function in the model organism *C. elegans*, and demonstrate differences in these parameters in nematodes carrying mutations in genes coding for proteins involved in fission, fusion, mitophagy, and the ETC that result in altered mitochondrial morphology. Interestingly, many strain differences were not apparent until chemical challenge, highlighting the importance of carrying out tests that incorporate both genetic differences and toxicant exposure before definitive conclusions can be drawn about overall mitochondrial function and resilience.
Table 1: Summary of mitochondrial parameters in fission, fusion, mitophagy mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aspect Ratio</th>
<th>Basal OCR (nmol/min/mg protein; P&lt;0.0001)</th>
<th>ATP Linked OCR (nmol/min/mg protein; P=0.65)</th>
<th>Maximal OCR (nmol/min/mg protein; P&lt;0.0001)</th>
<th>Spare Capacity (nmol/min/mg protein; P=0.022)</th>
<th>Proton Leak (nmol/min/mg protein; P&lt;0.0001)</th>
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<tbody>
<tr>
<td>N2</td>
<td></td>
<td>8.78±0.34 (n=37)</td>
<td>4.27±0.44 (n=12)</td>
<td>18.40±0.74 (n=13)</td>
<td>8.51±0.96 (n=13)</td>
<td>1.15±0.33 (n=8)</td>
</tr>
<tr>
<td>CU5991 fzo-1 (tm1133)</td>
<td>4.24±0.29 (n=147)</td>
<td>8.78±0.34 (n=37)</td>
<td>4.27±0.44 (n=12)</td>
<td>18.40±0.74 (n=13)</td>
<td>8.51±0.96 (n=13)</td>
<td>1.15±0.33 (n=8)</td>
</tr>
<tr>
<td>cat-3 (ad426)</td>
<td></td>
<td>8.74±0.28 (n=55; P=0.816)</td>
<td>6.24±0.50 (n=15)</td>
<td>13.40±0.72 (n=21; P&lt;0.0001)</td>
<td>4.64±0.91 (n=21; P=0.020)</td>
<td>0.34±0.18 (n=10; P=0.039)</td>
</tr>
<tr>
<td>CU6372 drp-1 (tm1108)</td>
<td></td>
<td>11.3±0.72 (n=52; P=0.0006)</td>
<td>3.98±0.95 (n=12)</td>
<td>15.20±1.01 (n=25; P&lt;0.0001)</td>
<td>4.50±1.30 (n=21; P=0.013)</td>
<td>1.83±0.20 (n=10; P=0.085)</td>
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<tr>
<td>pink-1 (tm1779)</td>
<td></td>
<td>9.47±0.46 (n=35; P=0.452)</td>
<td>4.16±0.76 (n=12)</td>
<td>19.1±1.99 (n=8; P=0.680)</td>
<td>8.21±1.23 (n=8; P=0.884)</td>
<td>-0.02±0.23 (n=10; P=0.004)</td>
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<tr>
<td>VC1024 pdr-1 (gk448)</td>
<td></td>
<td>10.20±0.55 (n=35; P=0.089)</td>
<td>4.13±0.37 (n=12)</td>
<td>19.3±2.94 (n=8; P=0.560)</td>
<td>9.13±2.56 (n=8; P=0.766)</td>
<td>1.78±0.31 (n=10; P=0.106)</td>
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<tr>
<td>MQ887 isp-1 (qm150)</td>
<td></td>
<td>8.29±0.38 (n=49; P=0.383)</td>
<td>5.06±0.59 (n=15)</td>
<td>15.40±0.75 (n=16; P=0.309)</td>
<td>6.75±0.71 (n=16; P=0.309)</td>
<td>2.32±0.32 (n=9; P=0.005)</td>
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</tbody>
</table>

*Values are shown as mean ± SE. ANOVA p-values are shown in parentheses under each column heading. N and p-values for post-hoc comparison to N2 nematodes in parentheses (when appropriate). **isp-1 mitochondria could not be quantified, likely due to poor MitoTracker Red CMXROS uptake due to decreased mitochondrial membrane potential (177, 178).
Figure 2: Response of L4 N2 nematodes to sodium azide alone and post-FCCP.

Response to sodium azide was assessed statistically with a one way ANOVA (P=0.0005). Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 3: Extracellular acidification rate in L4 N2 and fzo-1 nematodes.

One way ANOVA (P>0.05). (n=11). Bars ± SEM
Figure 4: Image processing result of a branched mitochondrion.

A centerline was extracted using a morphological thinning operation.
Figure 5: Oligomycin does not reduce OCR in L4 N2 nematodes.

Representative Seahorse output data. (n=4 for each concentration shown).
Figure 6: Titration of DCCD in L4 N2 nematodes.

Significance assessed with a one way ANOVA (P<0.0001), followed by student’s T-tests for pairwise comparisons. Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 7: Effect of DMSO on efficacy of 20µM DCCD.

Significance assessed with a one way ANOVA (P<0.0001), followed by student’s T-tests for pairwise comparisons. Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 8: Representative Seahorse XFe24 output data for L4 N2 nematodes dosed with (A) DCCD, (B) FCCP, and (C) sodium azide.
Figure 9: Oligomycin reduces OCR in cuticle-deficient bus-8 nematodes.

Both a (A) 12-hour pre-incubation with oligomycin (one way ANOVA, P<0.0001), and (B) injecting oligomycin directly into the Seahorse XFe24 (one way ANOVA, P=0.0007) reduced OCR in cuticle deficient bus-8 nematodes. Bars ± SEM.
Figure 10: DCCD titration with *bus*-8-deficient nematodes.

Treatment with DCCD caused a significant reduction in OCR (one way ANOVA, $P<0.0001$). Bars ± SEM.
Figure 11: Effect of 20µM DCCD and 50µM oligomycin in *bus*-8 nematodes.

20µM DCCD caused a significantly greater reduction in OCR than 50µM oligomycin in *bus*-8 nematodes (one way ANOVA, main effect of treatment P=0.0005). Bars ± SEM.
Figure 12: 2,4-Dinitrophenol fails to increase OCR in L4 N2 nematodes.
Figure 13: Titration of FCCP in L4 N2 nematodes.

Significance assessed with a one way ANOVA (main effect of treatment, P<0.0001). Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 14: Effect of DMSO concentration on efficacy of 15µM FCCP. Representative Seahorse XF24 output data. (n=4 for each concentration shown).
Figure 15: Titration of sodium azide in L4 N2 nematodes.

Significance assessed with a one way ANOVA (main effect of treatment, P<0.0001). Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 16: Basal OCR is elevated in L4 *drp-1* nematodes.

Statistical significance was analyzed via a one way ANOVA (main effect of strain, \( P<0.0001 \)) (\( n=31-45 \)). Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 17: The fundamental parameters of the mitochondrial respiratory chain are altered in eat-3 nematodes.

Panel A shows the overall Seahorse XFe respiratory profile for wild-type and eat-3-deficient nematodes. eat-3-deficient nematodes have reduced (A) basal OCR (P=0.0005), (B) ATP-linked OCR (P=0.0013), (D) maximal OCR (p<0.0001), and (E) spare respiratory capacity (P=0.0014), but not (F) proton leak (P=0.77), compared to wild-type nematodes. N=16-49. Asterisks (*) denote statistical significance (one way ANOVA) between N2 and eat-3. Bars ± SEM.
Figure 18: ATP coupled respiration.

(A) 20µM DCCD caused a significant reduction in OCR in all strains (two way ANOVA, main effects of strain and treatment, P<0.0001 for both, but not their interaction). (B) A trend in increased ATP coupled respiration was observed in fzo-1 nematodes (one way ANOVA, P=0.07). (n=12-16). Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 19: Maximal and spare respiratory capacity in L4 nematodes.

(A) Treatment with FCCP caused a significant increase in OCR in all strains (two way ANOVA, main effects of strain, treatment and their interaction, P<0.0001 for all); however, L4 fzo-1, drp-1 and isp-1 had reduced maximal respiratory capacity compared to N2 nematodes (Student’s t-test, p=0.03, p<0.0001, p=0.01, respectively). (B) Spare respiratory capacity was reduced in fzo-1 and drp-1, compared to wild-type nematodes (one way ANOVA, P=0.022). (n=12-20). Asterisks (*) denote statistical significance. Bars ± SEM.
DMSO had no effect on OCR in any of the strains tested (two way ANOVA, P>0.05). Bars ± SEM.
Sodium azide caused a significant reduction in OCR in all strains tested (one was ANOVA, $P<0.0001$). Bars ± SEM.
Figure 22: Proton leak in L4 nematodes.

(A) Sodium azide and DCCD caused significantly different reductions in OCR in N2, *drp-1*, *pink-1*, and *pdr-1* nematodes (two way ANOVA, main effects of strain (P<0.0001), treatment (P<0.0001) and their interaction (P=0.0002)), while *fzo-1* and *isp-1* responses were not significantly different. (B) L4 *fzo-1* and *isp-1* nematodes have reduce proton leak, while *pink-1* *C. elegans* have increased leak (one way ANOVA, P<0.0001). (n=8-12). Asterisks (*) denote statistical significance. Bars ± SEM.
Figure 23: Mitochondrial morphology in N2 and mutant C. elegans.

Representative confocal images of mitochondrial morphology in wild-type (N2) (A-B) and mitochondrial mutant strains (fzo-1 (C-D), drp-1 (E-F), pink-1 (G-H), pdr-1 (I-J)) at L4. Left panel shows the sample at 63x, right panel shows a representative zoomed and cropped image from the z-stack (63x) used for image analysis.
3. Deficiencies in mitochondrial dynamics sensitize Caenorhabditis elegans to arsenite and other mitochondrial toxicants by reducing mitochondrial adaptability

This chapter was submitted for publication under the same title. The authors are Anthony L. Luz, Tewodros R. Godebo, Latasha L. Smith, Tess C. Leuthner, Laura L. Maurer, and Joel N. Meyer.

3.1 Introduction

Mitochondria, best known for their role in ATP production, are dynamic organelles that fuse and divide in response to various cellular and environmental cues. These cues regulate mitochondrial shape, number, and size, as well as the ability to meet demand for cellular energy (35). Fission and fusion are mediated by guanosine triphosphatases in the dynamin family, whose combined action fuse and divide the mitochondrial double membrane. The process of fission is mediated by cytosolic dynamin-related protein-1 (DRP1), which is recruited to the outer mitochondrial membrane (OMM), forming a spiral that constricts upon GTP hydrolysis to drive scission of the organelle. Alternatively, membrane-anchored mitofusins (MFN1, MFN2) mediate fusion of the OMM, while OPA1 mediates fusion of the inner mitochondrial membrane (203).

In addition to governing mitochondrial morphology, fission and fusion also play an important role in mitochondrial stress response (204). For example, mitochondria
carrying low levels of damaged mitochondrial DNA (mtDNA) or proteins can fuse with healthy mitochondria allowing contents to mix, resulting in the generation of healthy mitochondria in a process known as functional complementation (38, 205). On the other hand, irreparably damaged mitochondria are hindered in rejoining the mitochondrial network through fusion, as a sustained loss of mitochondrial membrane potential triggers the proteolytic cleavage of OPA1 preventing inner membrane fusion, and thus preventing poisoning of the mitochondrial network (39). Damaged mitochondria can then be eliminated via mitophagy, as loss of membrane potential also results in the accumulation of PTEN-induced putative kinase 1 (PINK1) on the OMM. PINK1 phosphorylates OMM proteins, including MFN2, and recruits the E3 ubiquitin ligase parkin (PARK2) from the cytosol. Parkin then ubiquitinates OMM proteins targeting the mitochondrion for autophagic degradation (40, 41). The importance of fission, fusion, and mitophagy (collectively referred to herein as “mitochondrial dynamics”) is demonstrated by the fact that deficiencies in these processes cause human disease. For example, mutations in PINK1 and PARK2 cause familial Parkinson’s Disease (PD) (42), while mutations in OPA1 and MFN2 cause dominant optic atrophy (DOA) (43) and Charcot-Marie-Tooth neuropathy type 2A (CMT2A) (44), respectively. The population frequencies of pathological mutations of these genes are 1 in 10,000 - 50,000 for OPA1 (206, 207), and 1 in 7,500 for MFN2 (208, 209). The frequency of PINK1 and PARK2 mutations is unknown; however, PARK2 mutations account for up to 40% of early-onset
PD cases (210), while *PINK1* accounts for 5% (211). Mutations in *DRP1* are not commonly associated with disease, although a few reports of neurodegeneration and epilepsy have been published (45, 46).

Despite the clear importance of these gene mutations, only 10% of PD cases are linked to genetics (212), while the age of onset and severity of clinical manifestations can vary dramatically between patients suffering from CMT2A, even in patients carrying identical mutations (213, 214). These observations strongly suggest a role for the environment in the development and progression of certain mitochondrial diseases; however, the relationship remains poorly understood. To address this knowledge gap, we screened ten known and suspected mitochondrial toxicants (2,4-dinitrophenol (DNP), acetaldehyde, acrolein, aflatoxin B1 (AfB1), arsenite, cadmium, cisplatin, doxycycline, paraquat, rotenone) for exacerbation of mitochondrial dysfunction in fission (*drp-1*), fusion (*fzo-1, eat-3* (MFN2, OPA1 homologs, respectively))-, and mitophagy (*pink-1, pdr-1* (*PINK1, PARK2* homologs, respectively))-deficient *Caenorhabditis elegans*. *C. elegans* is a powerful *in vivo* model to study mitochondrial gene-environment interactions, as mitochondrial function and many biochemical pathways are well conserved with humans (108, 111-115). *In vivo* analysis is important, as mitochondrial function is dependent upon intercellular signals, many of which arise from diverse tissues and cell types, and are lost in *in vitro* models (135).
Here, we identify several novel gene-environment interactions. In particular, deficiencies in fission and fusion sensitized nematodes to the inorganic trivalent arsenical, arsenite. Of the toxicants tested, arsenite may have the highest potential for human health relevance, as over 140 million people worldwide are chronically exposed to arsenite via consumption of contaminated drinking water (215, 216). Although chronic arsenite exposure is associated with cancer (217-220), and other metabolism-related pathologies (221-223), the precise mechanisms underlying pathogenesis are complex, and remain poorly understood. We also demonstrate that arsenite disrupts mitochondrial energy metabolism in fusion (fzo-1, eat-3)-deficient nematodes, while increasing mitochondrial function in wild-type nematodes, and has minimal effect on mitochondrial function in fission (drp-1)-deficient nematodes. These results support the importance of mitochondrial disruption in arsenic’s mechanism of toxicity, highlight the critical role for mitochondrial dynamics in responding to exposure, and suggest that a significant fraction of the population may be especially vulnerable to arsenic toxicity due to genetic deficiencies in mitochondrial dynamics.

### 3.2 Methods

#### 3.2.1 C. elegans strains and culture conditions

Wild-type (N2 Bristol), VC1024 pdr-1 (gk448; outcrossed (OC) x 3), and SJ4103 (zcIs14 [myo-3::GFP (mit)]) nematodes were purchased from the Caenorhabditis Genetics Center (CGC, University of Minnesota). CU5991 fzo-1 (tm1133; OC x 4) and DA631 eat-3
(ad426; OC >1) were provided by Alexander Van der Bliek, University of California, CU6806 fzo-1 (tm1133); drp-1 (tm1108) and CU6372 drp-1 (tm1108; OC x 9) were provided by Ding Xue, University of Colorado, and pink-1 (tm1779; OC x 1) were provided by Guy Caldwell, University of Alabama. All strains are referred to elsewhere in this manuscript by their gene name.

Synchronous populations of larval stage 1 (L1) nematodes were generated via sodium hydroxide bleach treatment as previously described (147) followed by overnight incubation on an orbital shaker in complete K-medium (150µl 1M CaCl₂, 150µl 1M MgSO₄, 25µl 10mg/ml cholesterol, 50mL sterile K medium (2.36g KCl, 3g NaCl, 1L ddH₂O)) at 20°C. Synchronous populations of nematodes were then maintained on K-agar plates seeded with Escherichia coli OP50 at 20°C as previously described (149).

### 3.2.2 Larval growth

Larval growth was measured using the COPAS Biosort (Union Biometrica Inc., Somerville, MA) as previously described (138). Fifty L1 nematodes were loaded into each well of a 96-well plate (4-6 wells per strain per treatment) using the COPAS Biosort. The volume of each well was then brought to 100µl with EPA H₂O (150), UVC-killed UvrA (UVC sensitive E. coli strain due to a lack of nucleotide excision repair (224)), and toxicant. With the exception of sodium arsenite (Ricca Chemical Company), all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). L1 nematodes were exposed to DNP (0, 250, 500, 1000µM in 0.5% DMSO), acetaldehyde (0, 1, 5mM in
ddH₂O), acrolein (0, 150, 200, 250, 300µM in ddH₂O), AfB₁ (0, 1, 3, 10µM in 0.5% DMSO), arsenite (0, 400, 600, 800µM in ddH₂O), cadmium chloride (0, 20, 80, 150µM in ddH₂O), doxycycline (0, 2.5, 5, 7.5µg/ml in 0.5% DMSO), paraquat dichloride hydrate (0, 25, 50, 150µM in ddH₂O), rotenone (0, 250, 500, 750nM in 0.5% DMSO) or cisplatin (0, 10, 20, 40, 60µM in ddH₂O). Caution must be used when handling acetaldehyde and acrolein, as these compounds are highly volatile. In addition 2,4-DNP is highly ionized at neural pHs (based on a pKₐ of 4.11); thus, reducing pH of the dosing media may increase the potency of DNP, as unionized compounds penetrate the cuticle more efficiently.

Nematodes were allowed to develop at 20°C for 48h, and larval growth was determined using the COPAS Biosort (138). All time of flight (ToF) data, a surrogate for nematode length, was normalized to percent control for each strain prior to statistical analysis, as some of the strains develop at reduced rates compared to wild-type nematodes under control conditions. Cisplatin growth assays were performed using a combination of the COPAS Biosort and Fiji-based measurements of nematode images acquired using NIS-Elements BR software and a Nikon SMZ1500 stereomicroscope. Nematode length was measured in Fiji using the segmented line function with splice fitting. All experiments were repeated 2-3 separate times.

### 3.2.3 Reproduction

Nematode reproduction was determined as previously described (138). Briefly, three L4 nematodes were loaded into each well of a 96-well plate (4-6 wells per strain
per treatment) using a COPAS Biosort (Union Biometrica Inc., Somerville, MA). The final volume of each well was then brought to 100µl with EPA water, UV-killed UvrA, and arsenite to a final concentration of 0, 200, 400, or 600µM. Nematodes were then allowed to reproduce for 48h at 20°C, and progeny were counted using the COPAS Biosort. Data was normalized to percent control, as some strains lay eggs at a reduced rate and/or have reduced fecundity compared to wild-type nematodes. Experiments were repeated three separate times.

3.2.4 Lethality and rescue assays

Arsenite lethality assays. Ten L4, 8 or 12 day old N2, fzo-1, eat-3, and drp-1 nematodes were added to each well of a 96-well plate using a platinum worm pick (2 wells per treatment per strain). The volume of each well was then brought to 100µl with complete k-medium, UV-killed UvrA, and arsenite to a final concentration of 0, 50, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000µM. Nematodes were exposed to arsenite for 24h at 20°C, and then scored as dead if they failed to move after repeated probing with a platinum worm pick.

Antioxidant and 3-MA rescue assays. Nematodes were exposed to antioxidants or small molecule inhibitors at concentrations previously shown to have therapeutic effects in nematodes (77, 225, 226). Briefly, ten 7.5-day old nematodes were added to each well of a 96-well plate and exposed to 10mM 3-MA, 100µM deferoxamine, 40µM
trolox, 60µM N-acetyl cysteine, or 5µM mitoQ for 12 hours. Each strain was then exposed to its 24h LC₅₀ concentration of arsenite, and lethality was scored 24h later.

**Dichloroacetate rescue.** L1 stage nematodes were placed on k-agar plates containing 500µM DCA, a concentration that has previously been shown to increase steady-state ATP levels in nematodes (227), and allowed to develop at 20ºC to the L4 stage. Nematodes were then exposed to 0, 2, 3, or 6 mM arsenite in liquid (as described above), and scored for survival 24 hours later.

**Basal OCR rescues.** 7.5-day old nematodes were exposed to 5µM mitoQ or 10mM 3-MA for 12 hours, and then exposed to 100µM arsenite for 24h. Following arsenite exposure, basal OCR was measured using the Seahorse XF²4 Bioanalyzer as described in section 4.6.

**Secondary toxicant exposures.** Following 24h exposure to 100µM arsenite, nematodes were exposed to secondary heat (37ºC) or redox stress (100mM paraquat). Briefly, 10 nematodes were placed on a k-agar plate using a platinum worm pick and placed in a 37ºC incubator. Nematodes were scored (every 2 hours) as dead when they failed to move after repeated probing with a platinum worm pick. The paraquat lethality assay was scored once, after 24h liquid exposure to paraquat.

### 3.2.5 Lifespan

Nematode lifespan was determined following chronic, lifelong (initiated at L1) exposure to 0, 25, or 100µM arsenite, and after acute (24h) exposure to 100µM arsenite in
8 day old nematodes. Twenty-five nematodes were added to control or arsenite containing K-agar plates seeded with *E. coli* OP50 and allowed to develop. Nematodes were monitored daily, and were scored as dead when they failed to move in response to repeated probing with a platinum worm pick. Lifespan experiments were repeated 2-3 separate times.

### 3.2.6 Seahorse XF<sup>e</sup> analysis

Using the Seahorse XF<sup>e</sup>24 Extracellular Flux Analyzer (Seahorse Bioscience, Massachusetts, USA), basal oxygen consumption rate (OCR), maximal OCR, and spare respiratory capacity were measured as previously described (228, 229). Briefly, nematodes were resuspended in unbuffered EPA H<sub>2</sub>O to a final concentration of 1.0±0.2 nematodes per microliter. Forty to seventy-five (75 L4 vs. 40 eight- and twelve-day old) nematodes were then pipetted into each well of a Seahorse utility plate, and the final volume of each well was brought to 525µl with unbuffered EPA H<sub>2</sub>O. An aliquot of nematodes was then frozen for total protein determination via the bicinchoninic acid assay (ThermoFisher Scientific). Eight basal OCR measurements were taken prior to the injection of 25µM FCCP (mitochondrial uncoupler), and then an additional 8 OCR measurements were taken following FCCP injection. Seahorse experiments were repeated three separate times.
3.2.7 ATP determination

Relative steady-state ATP levels were determined as previously described (230). First, 200 L4, 50 8-day old, or 50 12-day old nematodes were loaded into a white 96-well plate (3 wells per strain per treatment) in 50µl k-medium, and then 50µl of Promega Mitochondrial ToxGlo assay medium (Promega, Madison, WI) was added to each well. Nematodes were then incubated at 20°C for 30 minutes, and then luminescence was measured using a FLUOstar Optima microplate reader equipped with a luminescence optic. Experiments were repeated 3-5 separate times.

3.2.8 Genome copy number determination

Mitochondrial (mtDNA) genome copy number was determined as previously described (231, 232). Briefly, six nematodes were added to 90µl proteinase K-containing lysis buffer using a platinum worm pick and frozen at -80°C. Samples were then thawed, and lysed by heating to 65°C for 1h. Crude nematode lysate was used as template DNA for RT-PCR based determination of mtDNA and nucDNA copy number. Standard curves were employed for determination of absolute copy number. Three samples per treatment per time point were collected for each experiment. Experiments were repeated two separate times.

3.2.9 Isolation of mitochondria and nematode lysate

Following arsenite exposure, nematodes were rinsed 2 times with cold MSM buffer (20.04g mannitol, 11.98g sucrose, 523.5mg MOPS, 0.5L milliQ H2O, pH 7.4),
transferred to a glass homogenizer and homogenized for 3 minutes. The homogenate was then transferred to an ultracentrifuge tube and spun at 300 x G for 10min at 4°C. The supernatant was then transferred to new tube and spun at 7000 x G for 10min at 4°C to yield the mitochondrial pellet. The supernatant was then discarded, and the pellet was resuspended in MSM buffer, and re-pelleted two additional times to rinse the mitochondrial fraction.

Following arsenite exposure, nematodes were rinsed two times with cold MSM buffer, transferred to a glass homogenizer and homogenized for 2min. The homogenate was then transferred to an ultracentrifuge tube and spun at 300 x G for 5min at 4°C to remove excess cuticle and large debris. The supernatant was then transferred to a new tube and spun at an additional two times to yield the crude nematode lysate.

3.2.10 Enzyme activity

Citrate synthase activity was measured in crude nematode lysate as detailed in (233, 234). Briefly, CHAPs (1% final concentration) was added to the crude lysate and allowed to sit on ice for 15min to solubilize the inner mitochondrial membrane. Samples were then spun at 14,000 RPM for 10min, and the resulting supernatant was used for measuring citrate synthase activity. 10µl (20-40µg protein) of the homogenate, 2µl 30mM acetyl-CoA, 2µl 10mM DTNM, and 176µl 100mM Tris HCl (pH 8) was pipetted into a clear 96 well plate, and allowed to sit at 25°C for 10min to initiate the reaction. Absorbance at 412nm was then measured every 20s for 3 minutes using a FLUOstar...
Optima microplate reader to measure the endogenous DTNM reduction. 10µl 10mM oxaloacetate was then added to each sample and absorbance at 412nm was measured every 20s for 5min. The change in absorbance over the linear range was calculated with endogenous DTNM reduction subtracted, and then normalized to total protein.

ETC CI activity was assayed in isolated mitochondria as previously described (235). Briefly, mitochondria were incubated in 20mM potassium phosphate buffer, 2.5mg/ml fatty-acid free BSA, and 2mM KCN at 20ºC for 10min. 2.0µl 5mM NADH and 2.0µl 2.5mM coenzyme Q₁ were then added to each well, and the rate NADH oxidation (Abs₃₄₀nm) was measured using a microplate reader. 40µM Rotenone was then added to each well to measure non-specific NADH oxidation.

PDH and IDH activity were measured in isolated mitochondria following 24h of exposure to 100µM arsenite using Colorimetric Assay Kits (BioVision, Cat. # K679 and K756). Mitochondria were isolated as described above, and resuspended in BioVision’s PDH or IDH Assay Buffer. PDH and IDH activity was then measured following the manufacturer’s instructions. All experiments were repeated 3 separate times.

3.2.11 Glycolysis assay

Glycolysis was assayed as described in (236). Briefly, 50 control or arsenite exposed nematodes were pipetted into each well (3 wells per treatment) of a white 96-well plate and exposed to EPA H₂O or 50mM 2-DG (final volume 50µl) for 4.5h at 20°C.
50µl of Promega Mitochondrial ToxGlo assay medium was then added to each well and steady-state ATP was determined as described in Section 3.2.7.

**3.2.12 Gene expression**

mRNA was isolated from nematodes using the Qiagen RNeasy Mini kit. 1500ng mRNA was then converted to cDNA using the Qiagen Omniscript Reverse Transcription kit. mRNA levels from control and arsenite treated nematodes were then measured using the 7300 Real-time PCR System.

Table 2 lists the annealing temperatures, amplicon sizes, and sequences of all PCR primers. The fold change of each gene was then calculated by comparing the cycle threshold (Ct) of the target gene to the Ct of the house keeping gene cdc-42 (237). Samples were collected in triplicate from two independent experiments.

**3.2.13 Mitochondrial morphology**

Control and arsenite exposed nematodes were exposed to 1µM Mitotracker Red CMXROS (Molecular Probes, Invitrogen) in complete K-medium overnight. Following Mitotracker exposure, nematodes were rinsed 2 times in fresh K-medium and incubated on an orbital shaker at 20°C for 30 minutes to allow excess dye to clear from their guts. Nematodes were then picked onto an agar pad containing levamisole (25 mg/mL) and subsequently imaged on a confocal microscope (Zeiss Axio 780 confocal upright with fixed stage) (228).
3.2.14 Arsenite analysis

Internal concentrations of arsenic were measured in isolated mitochondria and in the crude nematode lysate. Briefly, following isolation, samples were treated with concentrated HNO₃ acid (1.5 ml, 15 N), 0.5 ml of ddH₂O, and 5 drops of 30% H₂O₂. The samples were then heated progressively on a hotplate from 50 to 80°C for 12h, while regularly being degassed (after 30min cooling) until the digestion step was completed. 0.45ml aliquots of the digested samples were then analyzed for total arsenic via a VG Plasmaquad 3 inductively coupled plasma–mass spectrometer.

3.2.15 Statistical analysis

Statistical analysis was performed using JMP v11.0 software (SAS Institute). LC₅₀ values were calculated using Probit analysis. Survival curves were analyzed via the non-parametric Mantel cox test. All other data was initially assessed via one or two way ANOVA, and when warranted (p<0.05) post-hoc analysis was performed using Tukey’s HSD test.

3.3 Results

3.3.1 Deficiencies in mitochondrial dynamics generally sensitize, but in some cases protect, nematodes from environmental mitotoxicants.

We wished to test whether deficiency in mitodynamic processes would sensitize nematodes to exposure to chemicals that cause mitochondrial damage. As larval development is dependent upon proper mitochondrial function in nematodes (238, 239),
we tested ten known and suspected mitotoxicants (DNP, acetaldehyde, acrolein, AfB₁, arsenite, cadmium, cisplatin, doxycycline, paraquat, and rotenone) for exacerbation of larval growth delay in fission (drp-1)-, fusion (fzo-1, eat-3)-, and mitophagy (pdr-1, pink-1)-deficient nematodes using a COPAS Biosort-based larval growth assay (138, 240).

Results from the larval growth screen are presented in Table 3.

Deficiencies in mitochondrial fusion genes fzo-1 and eat-3 sensitized nematodes to arsenite (Figure 24A and Figure 25), aflatoxin B₁ (AfB₁) (Figure 26), paraquat (Figure 27), rotenone (Figure 28), 2,4-dinitrophenol (DNP) (Figure 29), and cisplatin (Figure 30) while only mildly sensitizing nematodes to acetaldehyde (Figure 31). Alternatively, deficiencies in mitochondrial fission (drp-1) only mildly sensitized nematodes to paraquat (Figure 27). Interestingly, deficiencies in pink-1 and pdr-1 resulted in varied responses to toxicants. pdr-1-deficient nematodes were mildly sensitized to rotenone (Figure 28), while pink-1 nematodes were sensitive to rotenone (Figure 28), and mildly sensitive to DNP (Figure 29), and doxycycline (Figure 32). Unexpectedly, resistance to several toxicants was observed. All fission-, fusion-, and mitophagy-deficient strains displayed resistance to acrolein (Figure 33), while only fzo-1 nematodes displayed resistance to cadmium (Figure 34), and pink-1 was mildly resistant to acetaldehyde (Figure 31).

Of the mitotoxicants tested, arsenite stood out as causing a large differential effect in multiple mitodynamics strains compared to wild-type. Furthermore, the highest
concentration of arsenite tested (800µM) induced significant lethality in \textit{fzo-1} and \textit{eat-3}, but not in other nematode strains. In addition, arsenic is of immense human health concern, as demonstrated both by the large number of people who are exposed due to drinking water contamination (described above), and by its importance at highly polluted sites around the United States (it was listed first on the Agency for Toxic Substances and Disease Registry’s 2015 substance priority list). Thus, arsenite was chosen for further mechanistic studies.

\subsection*{3.3.2 Deficiencies in mitochondrial fission and fusion sensitize nematodes to arsenite throughout aging.}

We hypothesized that fission and mitophagy deficiencies, which surprisingly did not result in sensitivity during our short-term larval growth assay, would be more deleterious over a longer time-course, as lack of these processes would inhibit removal of damaged mitochondria. To test this, we exposed L4 stage \textit{drp-1}, \textit{pink-1}, and \textit{pdr-1} nematodes to arsenite and monitored reproduction. Deficiencies in \textit{drp-1}, but not \textit{pdr-1} or \textit{pink-1} sensitized nematodes to arsenite-induced reproductive toxicity (Figure 24B). We therefore included \textit{drp-1}, but not \textit{pdr-1} or \textit{pink-1} in subsequent studies.

Next, to identify non-lethal arsenite exposure conditions and potential windows of sensitivity throughout aging, we performed acute and chronic arsenite exposures with young (L1-L4 stage), middle (8-day old) and old age (12-day old) nematodes. Chronic, lifelong exposure to 25µM arsenite did not affect nematode lifespan, while
100µM arsenite reduced the lifespan of all strains; however fzo-1, eat-3, and drp-1 were not significantly sensitive compared to wild-type (Figure 35 & Table 4). On the other hand, fzo-1 and eat-3 were hypersensitive to acute arsenite toxicity (24h lethality) throughout aging (L4, 8-, 12-days of age), while drp-1 were sensitive only later in life (8- and 12-days of age) compared wild-type nematodes (Figure 36). Sensitivity to arsenite-induced lethality increased dramatically (more than 10-fold) throughout aging in all strains, with 24h LC₅₀ values declining from 5.73 (N2), 2.76 (fzo-1), 2.97 (eat-3), 6.03 (drp-1) mM arsenite at the L4 stage to 0.64 (N2), 0.22 (fzo-1), 0.12 (eat-3), 0.38 (drp-1) mM arsenite at 12-days of age (Table 5).

3.3.3 Arsenite preferentially disrupts mitochondrial function in fusion-deficient nematodes throughout aging.

While previous research and our results up to this point provides evidence that arsenite targets mitochondria, they do not specifically demonstrate altered mitochondrial function, or strain-specific differences in the mitochondrial response to arsenic. Therefore, we next investigated the effects of non-lethal arsenite exposure (25, 100, or 1,500µM depending upon life stage) on mitochondrial function in L4 stage, 8- and 12-day old fzo-1, eat-3, drp-1, and wild-type nematodes. Arsenite reduced basal OCR and ATP content in L4 stage fzo-1 and eat-3 nematodes, while also reducing spare respiratory capacity (SRC) in N2, fzo-1, and eat-3 nematodes (Figure 37A-D). Unexpectedly, arsenite increased basal OCR, maximal OCR, and spare respiratory
capacity (trending increase, P=0.059 for post-hoc comparison) in 8-day old wild-type nematodes (Figure 37E-H), while reducing basal OCR, ATP, maximal OCR, and SRC in fusion (fzo-1, eat)-deficient nematodes (Figure 37E-H). Finally, at 12-days of age arsenite increased basal OCR in wild-type nematodes, decreased basal OCR in eat-3 and drp-1, had no effect on ATP or maximal OCR, and decreased SRC in fzo-1 (Figure 37I-L).

However, maximal OCR and SRC declined steadily throughout aging until SRC fell below detection limits in 12-day old eat-3 and drp-1 nematodes (Figure 37L); thus, these parameters must be interpreted with caution in aging old nematodes. P-values for statistical analysis are provided in Table 6.

Collectively, these results demonstrate that arsenic disrupted mitochondrial function in fusion-deficient nematodes, while having minimal adverse effects or even beneficial effects on mitochondrial function in wild-type and drp-1 nematodes. The lack of effect in drp-1 nematodes is particularly remarkable given their reduced basal SRC. Furthermore, these results also demonstrate an age-related increase in sensitivity to arsenite-induced mitochondrial dysfunction in fusion-deficient nematodes.

3.3.4 The role of mitochondrial morphology, autophagy, ROS, the unfolded protein response, and mitochondrial biogenesis in arsenite-induced toxicity.

Mitochondrial dynamics are integral to many homeostatic processes. To investigate which were responsible for the sensitivity of the fusion-deficient strains, we next investigated the role of mitochondrial morphology, autophagy, ROS, the unfolded
protein response, and mitochondrial biogenesis in arsenite-induced mitochondrial
dysfunction. Because development of the nematode germline at the L3-L4 transition is
associated with large increases in mtDNA copy number and OXPHOS (112, 151), which
would confound mechanistic studies, we performed all experiments in post-mitotic, 8-
day old nematodes, using a non-lethal 24h exposure to 100µM arsenite. This life stage
was also of particular interest because arsenite exposure at 8 days induced opposing
responses in wild-type and fusion-deficient nematodes, increasing mitochondrial
function in wild-type and disrupting mitochondrial function in fusion-deficient
nematodes (Figure 37E-H).

3.3.5 Mitochondrial morphology per se does not influence arsenite
sensitivity.

Because mitochondrial fusion facilitates functional complementation, we
hypothesized that arsenite would induce mitochondrial fusion in wild-type nematodes.
This would be consistent with fusion deficiency conferring sensitivity, since fzo-1 and
eat-3 nematodes would lack this protective response. Moreover, hyper-fused
mitochondria tend to be more metabolically active (36, 37); thus an increase in
mitochondrial fusion could also explain the increase in mitochondrial respiration
observed in wild-type nematodes. However, arsenite had no obvious effect on
mitochondrial morphology (representative images shown in Figure 38) or expression of
drp-1 or fzo-1 mRNA in wild-type nematodes (Figure 39), expression of which has
previously been shown to track well with toxicant-induced alterations in mitochondrial morphology (241). Next, as mitochondrial morphology is maintained through a balance of fission and fusion, and loss of both fission and fusion restores mitochondrial networks back to a wild-type morphology (179), we hypothesized that inhibition of \textit{drp-1} in \textit{fzo-1} or \textit{eat-3} nematodes would reduce sensitivity to arsenite. However, pharmacological inhibition of \textit{drp-1} with mdivi-1 (242, 243) in \textit{fzo-1} and \textit{eat-3} nematodes had no effect on mitochondrial morphology in either strain (data not shown). Finally, we tested L4 stage \textit{fzo-1; drp-1} double mutants for sensitivity to arsenite using a 24h lethality assay; however, \textit{fzo-1; drp-1} double mutants displayed similar sensitivity to arsenite as \textit{fzo-1} nematodes (Figure 40). Collectively, these results demonstrate that mitochondrial morphology alone does not dictate arsenite sensitivity, but support the importance of being capable of mounting a dynamic morphological response to stress (which is lost in the double mutant).

### 3.3.6 Autophagy has a limited role in arsenite toxicity.

Autophagy is often a protective response, but can in some cases cause toxicity. In response to starvation-induced autophagy, mitochondria upregulate fusion to elongate the mitochondrial network. Because elongated mitochondria are recalcitrant to autophagic degradation (244), increased fusion may represent a protective response that maintains mitochondrial mass, which would be lost in \textit{fzo-1} and \textit{eat-3} nematodes. As arsenite can induce autophagy in \textit{C. elegans} (245) and other mammalian models (246,
we hypothesized that arsenite-induced autophagy results in degradation of fragmented mitochondria in fusion-deficient nematodes resulting in reduced mitochondrial content and dysfunction. In partial agreement with this, pretreating fzo-1-deficient nematodes with the type III phosphatidylinositol 3-kinase inhibitor 3-methyladenine (3-MA) partially rescued nematodes from arsenite-induced lethality (Figure 41A); however, 3-MA pretreatment failed to prevent low-dose (100µM) arsenite from reducing basal OCR in fusion-deficient nematodes (Figure 41B). Furthermore, 100µM arsenite did not induce autophagy (lgg-1, atg-18) or mitophagy (dct-1) gene expression (Figure 42). Collectively, these results suggest that arsenite-induced autophagy may exacerbate lethality in fzo-1 at higher, acutely toxic concentrations; however, at lower, non-lethal concentrations, autophagy does not appear to play a role in inducing mitochondrial dysfunction.

### 3.3.7 Arsenite induces superoxide dismutase expression.

Generation of reactive oxygen species (ROS) is thought to be one of the principal mechanisms by which arsenic induces disease (248). As arsenite has been shown to induce ROS in C. elegans (245, 249), and as deficiencies in eat-3 have been shown to sensitize nematodes to ROS (250), we hypothesized that arsenite-induced ROS was contributing to the mitochondrial dysfunction observed in fusion-deficient nematodes. However, treatment with N-acetyl cysteine, trolox (a water-soluble vitamin E analog), deferoxamine (iron chelator that limits Fenton chemistry), or Mito-TEMPO
(mitochondrial-targeted SOD mimetic) failed to alter sensitivity to arsenite-induced lethality in any strain (Figure 43). On the other hand, treatment with the mitochondrial-targeted antioxidant mitoQ partially rescued lethality in eat-3 and drp-1 (Figure 44A); however, mitoQ increased basal OCR in fission- and fusion-deficient nematodes regardless of arsenite treatment, confounding results from an attempted mitoQ rescue of basal OCR (Figure 44B). Next, to gain insight into the specific intracellular compartments where superoxide is being generated we measured expression of cytosolic (sod-1), mitochondrial (sod-2, sod-3), and extracellular (sod-4) superoxide dismutase genes. Treatment with 100µM arsenite induced sod-1 in wild-type, fzo-1, and eat-3; had no effect on sod-2 expression; decreased sod-3 expression in wild-type and fzo-1; and increased sod-4 expression in fzo-1 nematodes (Figure 45). As expected, these results suggest a role for superoxide anion in arsenite toxicity; however, they are not supportive of mitochondria being the principle site of superoxide-generation.

3.3.8 Arsenite induces the unfolded protein response and disrupts pyruvate metabolism, with exacerbation of mitochondrial dysfunction specifically in fusion mutants.

The binding of protein sulfhydryl groups by arsenite is one of the principal mechanisms of toxicity of arsenite, resulting in enzyme inhibition and protein misfolding. An induction of the unfolded protein response (UPR) can serve to counteract this toxicity, but can also contribute to arsenite-induced toxicity and pathogenesis (249, 251). To test the potential protective or pathological roles of the UPR in this context, we
next measured cytosolic (hsp-16.2), endoplasmic reticulum (hsp-4), and mitochondrial (hsp-6) specific heat shock protein (HSP) expression. Arsenite induced hsp-16.2 in all strains, but hsp-4 only in fzo-1, and hsp-6 only in fzo-1 and eat-3 (Figure 46). These results confirm arsenite induces the UPR in C. elegans, and suggest a disruption specifically of mitochondrial proteostasis occurs at this level of exposure only in fzo-1 and eat-3.

To test whether specific disruption of mitochondrial enzymes is also more sensitive in fusion mutants, we compared inhibition of known targets of arsenite across strains. Arsenite inhibits enzymes involved in pyruvate metabolism and the Krebs cycle, including pyruvate, succinate, isocitrate, and α-ketoglutarate dehydrogenases (245, 252-254). We measured the activity of pyruvate (PDH) and isocitrate dehydrogenase (IDH); activity of both was inhibited by arsenite in fzo-1 and eat-3 (Figure 47), but not in drp-1 or wild-type nematodes. As compensatory shifts in metabolism from OXPHOS to aerobic glycolysis have been reported following arsenite exposure (154, 245), we next tested for sensitivity to the hexokinase inhibitor 2-deoxy-D-glucose (2-DG) and measured glyceraldehyde 3-phosphate dehydrogenase expression (gpd-3, catalyzes the 6th step of glycolysis), which has previously been shown to be an inducible marker of glycolysis in nematodes (255). Arsenite exposure did not sensitize any strain to 2-DG-induced reductions in steady-state ATP levels (Figure 48A), nor did arsenite alter gpd-3 expression in fission- or fusion-deficient nematodes (Figure 48B). However, a reduction in gpd-3 expression was observed in wild-type nematodes. Collectively, these results
suggest that disruption of pyruvate metabolism and Krebs cycle activity underlie arsenite-induced mitochondrial dysfunction in *fzo-1* and *eat-3* nematodes.

### 3.3.9 Cellular and organismal excretion of arsenic

As extrusion of arsenite via the ArsA ATPase (*asna-1*) is one mechanism by which nematodes detoxify arsenic (256), we hypothesized that reduced mitochondrial function in fission- and fusion-deficient nematodes may limit arsenite excretion in these metabolically compromised strains. In partial agreement with this, arsenite accumulated in crude nematode lysate isolated from *drp-1*, but not *fzo-1* or *eat-3*, compared to wild-type nematodes (Figure 49A); however, arsenite did not differentially accumulate in isolated mitochondria (Figure 49B), nor did arsenite exposure significantly affect *asna-1* expression (Figure 50A). To further test the role of energetics in limiting arsenite excretion we treated nematodes with the PDH kinase (a negative regulator of PDH) inhibitor dichloroacetate (DCA), which has previously been shown to increase steady-state ATP levels and reduce lactic acidosis in wild-type and *fzo-1* nematodes, respectively (156, 227). In agreement with previous reports, DCA treatment increased steady-state ATP levels (Figure 50B); however, DCA treatment failed to reduce sensitivity to arsenite-induced lethality in any strain (Figure 50C). These results suggest that steady-state ATP levels are not rate limiting in the excretion of arsenite by the ArsA ATPase in nematodes. However, it is unclear if DCA treatment can overcome PDH
inhibition by arsenite in fusion-deficient nematode strains, although PDH inhibition was not observed in wild-type nematode strains.

3.3.10 Arsenite increases mitochondrial function in wild-type nematodes.

Unexpectedly, arsenite increased mitochondrial function in 8-day old wild-type (but not mutant) nematodes (Figure 37E-H). This could not be attributed to mitochondrial fusion, a process associated with increased metabolic activity, since no increase in mitochondrial fusion was observed (Figure 38 & Figure 39). To test the alternate hypothesis that increased oxygen consumption resulted from mitochondrial biogenesis, we measured the expression of mitochondrial DNA polymerase $\gamma$ (polg-1, the sole replicative mitochondrial DNA polymerase), the C. elegans mitochondrial transcription factor A (TFAM) ortholog (hmg-5), which is required for mtDNA replication and transcription (257), and mtDNA encoded nd-5 (ETC CI) and ctb-1 (ETC CIII). However, arsenite did not significantly alter the expression of polg-1 or hmg-5 in any strain (Figure 51). Arsenite did induce a small, but significant increase in nd-5 expression in eat-3, while mildly reducing ctb-1 expression in wild-type nematodes (Figure 51). As a clear transcriptional response for biogenesis was not observed, we next quantified mitochondrial mass in body wall muscle cells using transgenic nematodes expressing mitochondrial-targeted GFP under the control of the body wall muscle specific promoter myo-3. Arsenite did not increase GFP expression in body wall muscle
cells (Figure 52); however, GFP expression may not be induced via the myo-3 promoter in the context of biogenesis, instead GFP may simply diffuse into more mitochondria resulting in no net change of GFP expression. Next, we measured whole worm citrate synthase (CS) activity, a frequently used biomarker of mitochondrial content (258), and observed a large (~45%) increase in CS activity in wild-type, but not mutant, nematodes following arsenite exposure (Figure 53A). Furthermore, arsenite increased ETC CI activity in wild-type nematodes (Figure 53B); while a non-significant trend in increased mtDNA content was also observed (Figure 53C). Collectively, these results confirm that arsenite increases mitochondrial function in wild-type nematodes, but not fusion-deficient strains. However, this effect either occurs without an increase in mitochondrial mass, or is limited to specific tissues, as no increase in body wall muscle GFP was observed.

3.3.11 Increased respiration associated with decreased, not increased, stress resistance.

Chronic, larval, low-dose (100nM) exposure to arsenite has previously been shown to extend nematode lifespan, increase stress resistance, increase mitochondrial respiration, and upregulate mitochondrial protein content (23), a phenomenon termed mitohormesis. However, the arsenite-induced increase in mitochondrial function in wild-type nematodes in the current study (which employs a higher exposure concentration (100µM) in 8 day old adults) does not appear to represent a horismic
response, as no increase in lifespan was observed in any strain (Figure 54 & Table 7), nor did arsenite exposure increase stress resistance. Instead, arsenite sensitized fission, fusion, and wild-type nematodes to the free radical generator paraquat (Figure 55A), and eat-3 and drp-1 deficient nematodes to thermal stress (Figure 55B). Furthermore, the observed enhancement of mitochondrial function is no longer apparent after longer (48h) arsenite exposures in wild-type nematodes (Figure 56). Instead, early signs of mitochondrial dysfunction (reduced maximal OCR, and trending reduction in SRC) begin to become apparent after longer arsenite exposures.

3.4. Discussion

3.4.1 Gene-environment interactions between mitochondrial dynamics genes and mitotoxicants are complex.

We initially hypothesized that deficiencies in mitochondrial dynamics, processes critical for mitochondrial function and stress response, would universally sensitize nematodes to toxicants that target mitochondria. While we did uncover strong, novel gene-environment interactions, the nature of those interactions was complex. Mitochondrial fusion (fzo-1, eat-3)-deficient nematodes were hyper-sensitive to the most toxicants, while mitophagy- and fission-deficient nematodes were sensitive to fewer toxicants, and in most cases where sensitivity was observed, the sensitivity was quantitatively less. The differences may result to some degree from the fact that the chemicals we chose have different mitochondrial mechanisms of toxicity, as well as
different non-mitochondrial effects. Rotenone is an ETC CI inhibitor, DNP is a mitochondrial uncoupler, paraquat is a redox cycler, cisplatin can induce irreparable mtDNA damage (259), AfB1 is a genotoxin capable of inducing irreparable mtDNA damage (68, 87), while arsenite can induce ROS and inhibit enzymes (248, 251, 260).

Results are further complicated by the fact that many of the tested genes have additional roles beyond fission, fusion, and mitophagy. For example, pink-1 can regulate ETC CI activity through phosphorylation (48), which may explain why pink-1-, but not pdr-1-deficient nematodes are hypersensitive to rotenone. A limitation to our chemical screen is that we limited the screen to larval growth; longer-term analyses could potentially uncover other sensitivities if, for example, effects of fission or mitophagy deficiency manifest over a longer time-course, or if gene-environment interactions become more important later in life, as was the case for drp-1 and arsenite. Furthermore, fission- and fusion-deficient nematode strains grow at reduced rates compared to wild-type and mitophagy-deficient strains, which reduces the dynamic range of larval growth inhibition. Thus, results must be interpreted with caution, particularly with observed cases of resistance. For example, complete L1 growth arrest results in a 75% reduction in growth in wild-type nematodes, while only a ~60% reduction in growth would be observed for fusion-deficient strains giving the false impression of toxicant resistance.

Overall, these results strongly support the importance of mitochondrial fusion in limiting mitochondrial toxicity. These results are consistent with emerging literature, as
exposure to manganese, 6-hydroxydopamine, or glutamate induce mitochondrial fission and dysfunction; however, inhibiting fission prevents toxicity (261-263). One explanation for this is that mitochondrial fusion promotes functional complementation, which can limit the impact of toxicant-induced damage. In further support of this, we have previously reported hyper-fused mitochondrial networks in *drp-1-, pink-1-,* and *pdr-1-*deficient nematodes (228); thus, it is conceivable that the hyper-fused phenotype provides buffering capacity against damage, helping to explain why more severe toxicant responses were not observed in fission- and mitophagy-deficient nematodes. We have previously noted similar patterns of strong sensitivity of fusion mutants, but mild sensitivity of fission and mitophagy mutants, in response to persistent mitochondrial DNA damage induced by ultraviolet light (76, 77). Alternatively, mitophagy is a biologically slow process compared to fission, fusion, and functional complementation (264, 265). Severely damaged mitochondria are initially prevented from rejoining the mitochondrial network through the proteasomal cleavage of OPA1 (*eat-3* homolog), thus preventing fusion prior to mitophagy (39). The slower kinetics of mitophagy may explain why mitophagy-deficient nematodes did not display higher sensitivity to toxicants, as initial damage can be limited via more rapid stress response pathways. On the other hand, phosphorylation of MFN2 (*fzo-1* homolog) by *pink-1* is required for the recruitment of parkin and subsequent mitophagy (51); thus, impaired
mitophagy may also contribute to the sensitivity of \textit{fzo-1}-deficient nematodes to some toxicants.

Unexpectedly, fission-, fusion-, and mitophagy-deficient nematodes displayed resistance to acrolein, a highly reactive air pollutant capable of inducing irreparable mtDNA damage and mutations \cite{266, 267}. However, resistance to acrolein has been observed in \textit{D. melanogaster} strains with reduced metabolic rate, which is likely due to reduced toxicant uptake due to diminished oxygen consumption \cite{268}. As we and other have shown altered metabolism in fission, fusion, and mitophagy-deficient nematodes \cite{192, 228}, it is likely that similar mechanisms underlie acrolein resistance in nematodes. Resistance to cadmium, a metal capable of inhibiting mitochondrial function through protein thiol binding \cite{83, 269-271}, was also observed in \textit{fzo-1}. In addition to fusion, MFN2 (\textit{fzo-1} homolog) also plays a role in tethering mitochondria to the endoplasmic reticulum (ER) allowing redox and calcium signaling to occur between the two organelles \cite{272}. As the ER is a major site of calcium storage, and is a target of cadmium toxicity due to molecular mimicry \cite{271}, it is possible that impaired ER-mitochondrial interactions due to a loss of \textit{fzo-1} would reduce the inadvertent transfer of cadmium from the ER to the mitochondria resulting in the observed resistance.
3.4.2 Arsenite increases mitochondrial function in wild-type but not fusion-deficient nematodes.

Arsenite increased mitochondrial function in 8-day old wild-type nematodes, causing an increase in basal OCR, maximal OCR, spare respiratory capacity (trending increase), citrate synthase and ETC CI activity. Both CS and ETC CI activity have been shown to correlate well with mitochondrial content (258), which suggests an induction of mitochondrial biogenesis. Interestingly, arsenite can also induce mitochondrial biogenesis and enhance mitochondrial function in human keratinocytes through an upregulation of TFAM (273). However, under the conditions of our experiments, arsenite did not detectably alter expression of mitochondrial biogenesis genes (*polg-1, hmg-5*) or mtDNA-specific transcripts (*nd-5, ctb-1*), nor did arsenite induce mitochondrial fusion, a process also associated with increased metabolic activity (36, 37), or increase expression of mitochondrial targeted GFP in body wall muscle cells. These conflicting results suggest increased mitochondrial function may not be due to increased biogenesis, but rather due to some other as-yet unidentified mechanism. For example, post-translation modifications (274) or positive allosteric regulation (275, 276) of OXPHOS may be playing a role; however, additional work is required to test this.

Interestingly, arsenite did not increase mitochondrial function in L4 stage or 12-day old wild-type nematodes. This may be explained by the age-related increase in sensitivity to arsenite. Furthermore, the range of concentrations in which arsenite
enhances mitochondrial function in keratinocytes is narrow (273); thus, making it conceivable that other, untested, concentrations of arsenite may enhance mitochondrial function at in young (L4 stage) or old age (12-day old) worms.

Emerging evidence suggests arsenite can induce hormesis at low doses. Evidence for arsenite-induced hormesis includes reduced risk of developing skin cancer in Denmark (277), increased growth advantage for cells in culture (23, 278), and lifespan extension, stress resistance, and increased mitochondrial respiration in *C. elegans* (23). However, we do not interpret the increase in mitochondrial function that we observed in wild-type nematodes as a hormetic response. First, the concentrations of arsenite used in the present study are high (100µM ≈ 7.5ppm) compared to concentrations of arsenite previously found to induce hormesis in nematodes (100nM arsenite), and the exposure was performed with 8-day old nematodes, not larvae, the lifestage in which hormetic effects are most frequently tested in *C. elegans* (23). Second, increased stress resistance did not accompany the enhancement in mitochondrial function. Instead, arsenite exposure increased sensitivity to the redox stressor paraquat. Third, patients with arsenic-induced Bowen’s disease (BD; cutaneous carcinoma) exhibit increased mitochondrial biogenesis compared to both healthy and non-arsenic-induced BD patients, and enhanced mitochondrial function provides a proliferative advantage for malignant cells (273). Thus, increased mitochondrial function in wild-type nematodes may represent an aberrant change in metabolism preceding pathogenesis. However, it is
also possible that increased mitochondrial function is part of the nematode stress response. In support of this, nematodes can extrude arsenite via the ArsA ATPase (256). Although stimulating pyruvate metabolism with DCA treatment did not reduce sensitivity to arsenite, it is unclear if ATP is rate limiting or if DCA treatment can overcome arsenite-induced PDH inhibition; thus, we cannot fully exclude this mechanism.

Importantly, the internal dose of arsenite measured in wild-type nematodes (69.5µg/L) is within the range (2.9 – 74.6µg/L) measured in blood samples taken from humans exposed to arsenic-contaminated drinking water (279, 280). The discrepancy between the high arsenite concentrations in the dosing media and internal concentrations is likely due to the nematode cuticle, a collagenous barrier that can limit uptake of certain compounds (163, 281).

Finally, irrespective of whether or not the term “hormesis” could be applied in our experiments with wild-type nematodes, it was striking that none of the potentially beneficial changes were observed in the fusion-deficient nematodes, which were sensitive to arsenite toxicity. This supports the idea that the process of mitochondrial fusion is a critical mechanism of mitochondrial stress resistance, an idea further supported by other findings in this study.
3.4.3 Deficiencies in mitochondrial fusion sensitize C. elegans to arsenite-induced mitochondrial dysfunction.

Mitochondrial fission and fusion are dynamic processes that control mitochondrial morphology and stress response (35). Interestingly, fusion-deficient nematodes were sensitive to arsenite in all toxicity assays (growth, lethality, mitochondrial dysfunction) and at all life stages (L1, L4, 8- and 12-days of age), while sensitivity appears to be life stage specific for *drp-1* nematodes, or may require more time to manifest. Furthermore, sensitivity to arsenite increased, relative to wild-type, as *drp-1* nematodes age. This is demonstrated by the fact that no sensitivity to arsenite was observed for *drp-1* in larval growth or L4 stage lethality assays (24h exposure), while sensitivity was observed in reproduction (48h L4 exposure), and lethality assays at 8 and 12 days of age. Furthermore, arsenite only induced mitochondrial dysfunction in 12-day old *drp-1* nematodes, despite the fact that 8-day old *drp-1* nematodes had higher intracellular arsenite concentrations compared to wild-type nematodes. The apparent age-dependent increase in sensitivity to arsenite may be explained by the fact that deficiencies in mitochondrial fission can result in impaired mitophagy and accumulation of damaged macromolecules (41, 77). Thus, the natural buffering effect that accompanies hyperfusion in *drp-1* nematodes may be lost as nematodes accumulate damage throughout aging, and may help to explain the age-related increase in arsenite sensitivity.
Despite decades of research, the precise mechanisms underlying arsenic-induced disease remain poorly understood; however, generation of ROS (248), enzyme inhibition by sulfhydryl group binding (251), and alterations in DNA methylation (260) are all believed to play a role. In agreement with previous reports (245, 249, 282), arsenite induced the expression of superoxide dismutase and heat-shock protein genes, suggesting a role for ROS and protein misfolding in arsenite toxicity in nematodes. Interestingly, arsenite only induced mitochondrial-specific hsp-6 expression in fzo-1 and eat-3 nematodes, which suggests a disruption of mitochondrial proteostasis. In agreement with this, arsenite inhibited both pyruvate (PDH) and isocitrate (IDH) dehydrogenase activity in fzo-1- and eat-3-deficient nematodes, suggesting a disruption of pyruvate metabolism and Krebs cycle activity. As the Krebs cycle is major source of the reducing equivalents NADH and FADH$_2$, which feed electrons into complex I and complex II of the ETC, respectively, disruption of the Krebs cycle may cause mitochondrial dysfunction in fusion-deficient nematodes through substrate limitation. Alternatively, a cytosolic disruption of nuclear-encoded mitochondrial-bound proteins may be occurring, which could lead to impaired protein import and mitochondrial dysfunction.

Collectively, our results and others’ suggest that mitochondrial proteins are one of many targets of arsenite, but that deficiency in mitochondrial fusion sensitizes by elevating the relative importance of those targets by reducing the adaptive
mitochondrial stress response. Previously we have shown that longer exposures to similar concentrations of arsenite (48h exposure to 50-500µM) disrupt pyruvate metabolism, induce severe mitochondrial dysfunction, and increase glycolysis in germline-deficient, but otherwise wild-type nematode strains (245). Furthermore, mild mitochondrial dysfunction is observed in wild-type nematodes following longer (48h) arsenite exposures. This supports the idea that the mechanism by which arsenite induces mitochondrial dysfunction (i.e. disruption of pyruvate metabolism and Krebs cycle activity) in fzo-1, eat-3, wild-type, and germline-deficient nematodes is conserved, but that mitodynamic deficiency effectively lowers the dose-response. Furthermore, no evidence of a compensatory increase in glycolysis was observed in fusion-deficient nematodes. Fragmented mitochondrial networks per se do not appear to be the main driver of toxicity, as fzo-1; drp-1 double mutants in which mitochondrial morphology is restored to a wild-type phenotype (179) display similar sensitivity to arsenite as fzo-1 nematodes. This suggests that it is the capacity for a dynamic response itself that permits an adaptive response. While additional work will be required to fully elucidate the mechanisms of this response, it is probable that a combination of factors, including impaired damage removal (77), a loss of functional complementation, and reduced metabolic plasticity (192, 228), all contribute to arsenite sensitivity in fusion-deficient nematodes. Most importantly, these results collectively suggest that individuals carrying mutations in mitochondrial fusion genes (OPA1, MFN2) may be especially sensitive to
arsenite toxicity, and warrant further investigation among people exposed to arsenic and other mitotoxicants.
Table 2: Primers for real-time PCR analysis of mRNA expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperature (C)</th>
<th>Primers (5’-3’)</th>
</tr>
</thead>
</table>
| cdc-42 | 111                | 60                        | F: GAG AAA AAT GGG TGC CTG AA  
|        |                    |                            | R: CTC GAG CAT TCC TGG ATC AT                                                   |
| sod-1  | 125                | 60                        | F: AAA ATG TCG AAC CGT GCT GT  
|        |                    |                            | R: CCG GGA GTA AGT CCC TGG AT                                                   |
| sod-2  | 194                | 60                        | F: CTC GCT GCC AGA TTT ACC AT  
|        |                    |                            | R: TGA ACT TGA GAG CTG GCT GA                                                   |
| sod-3  | 212                | 60                        | F: GCA ATC TAC TGC TCG CAC TG  
|        |                    |                            | R: CAG CCT CGT GAA GTT TCT CC                                                   |
| sod-4  | 189                | 60                        | F: GCA CGT GCC TAC ATT TTC AA  
|        |                    |                            | R: TCC ACC AGC ACT TAG ACA ACC                                                   |
| hsp-4  | 138                | 56                        | F: CGT TCA AGA TCG ACA AGT  
|        |                    |                            | R: GAC CAA GGT AGG ATT CGG CA                                                   |
| hsp-6  | 76                 | 56                        | F: TCG TGT CAT CAA CGA GCC AA  
|        |                    |                            | R: AGC GAT CTT ATC TCC AGC G                                                   |
| hsp-16.2| 60                 | 56                        | F: CGC CAA AGA AAG CGG TT  
|        |                    |                            | R: CTT CGA TTT CCT GT TGA                                                       |
| polg-1 | 95                 | 60                        | F: CTG CCT AAT ACC GTT GCC TTT TT  
|        |                    |                            | R: AAT CCG GAC GCC TCC AA                                                       |
| hmg-5  | 108                | 60                        | F: TGC TGT CAT CAA CGA GCC AA  
|        |                    |                            | R: GGC CCA AAG TCA AAG CTC CA TAA TTA AAA AAC C                                 |
| nd-5   | 88                 | 60                        | F: TTA GCA AGT TTG GTC GAA GATT  
|        |                    |                            | R: GCC CCA AAG TAA CTA TTG AAA AAC C                                            |
| ctb-1  | 116                | 60                        | F: TTC CAA TTT GAG GGC CAA CT  
|        |                    |                            | R: AACTAGAATAGC TCA CGG CAA TAA AAA                                             |
| asna-1 | 116                | 60                        | F: TGT TTG GAA TGG GAG GTG GA  
|        |                    |                            | R: GAC GAA TGT GGT GCA ATG AGG A                                               |
| fzo-1  | 79                 | 60                        | F: GTC CCA TTT GTC CAA TAT AGT T  
|        |                    |                            | R: TGG GAC GAA TTA TTG CTG GT                                                   |
| drp-1  | 99                 | 60                        | F: GTC AGC TGT ACA AAC CCG AC  
|        |                    |                            | R: GGA GGG CCT TCA TAG TTT CAA G                                                |
| gpd-3  | 104                | 59                        | F: CGA GGA CGT TCC CAG TCA CGG A  
|        |                    |                            | R: CCG TGT CAT CCA GGT AGT TGT AGA CCT T                                        |
| lgg-1  | 196                | 60                        | F: GCA CGA AAG TCA AAG CTC CA  
|        |                    |                            | R: CCT GTG CAT GGT CCT GTG AG                                                    |
| atg-18 | 94                 | 60                        | F: TGG GAC ACA AAG ATG GCT A  
|        |                    |                            | R: CCA AGA TGT GTA AGA TTT TCG CC                                               |
| dct-1  | 145                | 60                        | For: ATC GCA CAA TCT CCT CAC GT  
|        |                    |                            | Rev: GGA CAG TCT TTG GAG GTG TAT T                                              |
Table 3: Results from toxicant larval growth screen.

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>N2</th>
<th>fzo-1</th>
<th>eat-3</th>
<th>drp-1</th>
<th>pink-1</th>
<th>pdr-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-DNP</td>
<td>96.0 ± 1.1%</td>
<td>83.8 ± 1.3% (p&lt;0.0001)</td>
<td>84.3 ± 1.4% (p&lt;0.0001)</td>
<td>88.9 ± 1.3% (p=0.0001)</td>
<td>87.6 ± 1.4% (p&lt;0.0001)</td>
<td>90.0 ± 1.5% (p=0.003)</td>
</tr>
<tr>
<td>Figure 29 (p&lt;0.0001)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Acetaldehyde</td>
<td>77.2 ± 0.8%</td>
<td>71.8 ± 0.8% (p&lt;0.0001)</td>
<td>70.6 ± 0.7% (p=0.0001)</td>
<td>76.5 ± 0.7% (p=0.95)</td>
<td>82.0 ± 0.8% (p&lt;0.0001)</td>
<td>76.7 ± 0.8% (p=0.99)</td>
</tr>
<tr>
<td>Figure 31 (p&lt;0.0001)</td>
<td></td>
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</tr>
<tr>
<td>Acrolein</td>
<td>74.6 ± 0.8%</td>
<td>88.1 ± 0.8% (p&lt;0.0001)</td>
<td>88.0 ± 0.8% (p&lt;0.0001)</td>
<td>80.9 ± 0.8% (p&lt;0.0001)</td>
<td>90.0 ± 0.8% (p&lt;0.0001)</td>
<td>83.5 ± 0.8% (p&lt;0.0001)</td>
</tr>
<tr>
<td>Figure 33 (p&lt;0.0001)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>90.8 ± 0.9%</td>
<td>69.1 ± 1.0% (p&lt;0.0001)</td>
<td>79.9 ± 0.8% (p&lt;0.0001)</td>
<td>87.3 ± 0.9% (p=0.051)</td>
<td>93.5 ± 1.6% (p=0.26)</td>
<td>90.9 ± 1.0% (p=1.00)</td>
</tr>
<tr>
<td>Figure 26 (p&lt;0.0001)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Arsenite</td>
<td>90.5 ± 0.7%</td>
<td>66.2 ± 0.7% (p&lt;0.0001)</td>
<td>65.2 ± 0.7% (p&lt;0.0001)</td>
<td>87.4 ± 1.2% (p=0.06)</td>
<td>87.8 ± 1.2% (p=0.10)</td>
<td>93.3 ± 1.1% (p=0.08)</td>
</tr>
<tr>
<td>Figure 24 (p&lt;0.0001)</td>
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</tr>
<tr>
<td>Cadmium</td>
<td>44.7 ± 0.7%</td>
<td>60.5 ± 0.7% (p&lt;0.0001)</td>
<td>49.2 ± 0.7% (p&lt;0.0001)</td>
<td>48.8 ± 0.7% (p&lt;0.0001)</td>
<td>41.3 ± 0.5% (p=0.0007)</td>
<td>44.0 ± 0.6% (p=0.89)</td>
</tr>
<tr>
<td>Figure 34 (p&lt;0.0001)</td>
<td></td>
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</tr>
<tr>
<td>Cisplatin</td>
<td>94.6 ± 0.7%</td>
<td>65.6 ± 1.7% (p&lt;0.0001)</td>
<td>79.9 ± 1.3% (p&lt;0.0001)</td>
<td>90.9 ± 1.0% (p=0.04)</td>
<td>94.3 ± 0.8% (p=0.99)</td>
<td>93.1 ± 0.8% (p=0.72)</td>
</tr>
<tr>
<td>Figure 30 (p&lt;0.0001)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>75.0 ± 1.1%</td>
<td>80.2 ± 1.2% (p=0.003)</td>
<td>80.2 ± 1.0% (p=0.003)</td>
<td>77.5 ± 1.2% (p=0.33)</td>
<td>68.4 ± 1.0% (p&lt;0.0001)</td>
<td>78.2 ± 1.1% (p=0.13)</td>
</tr>
<tr>
<td>Figure 32 (p&lt;0.0001)</td>
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<td></td>
</tr>
<tr>
<td>Paraquat</td>
<td>87.6 ± 1.0%</td>
<td>78.5 ± 0.9% (p&lt;0.0001)</td>
<td>72.1 ± 0.8% (p&lt;0.0001)</td>
<td>82.7 ± 0.9% (p=0.0005)</td>
<td>84.6 ± 0.8% (p=0.07)</td>
<td>86.1 ± 0.9% (p=0.65)</td>
</tr>
<tr>
<td>Figure 27 (p&lt;0.0001)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotenone</td>
<td>68.0 ± 0.8%</td>
<td>53.7 ± 0.7% (p&lt;0.0001)</td>
<td>54.3 ± 0.5% (p&lt;0.0001)</td>
<td>69.6 ± 0.8% (p=0.31)</td>
<td>50.6 ± 0.6% (p&lt;0.0001)</td>
<td>62.3 ± 0.7% (p&lt;0.0001)</td>
</tr>
<tr>
<td>Figure 28 (p&lt;0.0001)</td>
<td></td>
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</tr>
</tbody>
</table>

Data is presented as the pooled effect of each toxicant on each nematode strains growth relative to control (percent control ± SEM). Each toxicant was initially analyzed via one way ANOVA (p-values in toxicant column). Post-hoc analysis of each fission, fusion, or mitophagy strain compared to N2 was performed using Dunnett’s test.
Table 4: Summary of lifespan analysis.

<table>
<thead>
<tr>
<th>Strain / treatment</th>
<th>Mean LS (Days ± SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 control</td>
<td>14.46 ± 0.43</td>
<td>NA</td>
</tr>
<tr>
<td>N2 + 25μM iAs³⁺</td>
<td>14.23 ± 0.46</td>
<td>0.83</td>
</tr>
<tr>
<td>N2 + 100μM iAs³⁺</td>
<td>11.86 ± 0.36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>fzo-1 control</td>
<td>13.60 ± 0.73</td>
<td>NA</td>
</tr>
<tr>
<td>fzo-1 + 25μM iAs³⁺</td>
<td>12.65 ± 0.75</td>
<td>0.41</td>
</tr>
<tr>
<td>fzo-1 + 100μM iAs³⁺</td>
<td>10.15 ± 0.52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>eat-3 control</td>
<td>15.51 ± 0.54</td>
<td>NA</td>
</tr>
<tr>
<td>eat-3 + 25μM iAs³⁺</td>
<td>15.73 ± 0.66</td>
<td>0.30</td>
</tr>
<tr>
<td>eat-3 + 100μM iAs³⁺</td>
<td>12.83 ± 0.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>drp-1 control</td>
<td>14.63 ± 0.51</td>
<td>NA</td>
</tr>
<tr>
<td>drp-1 + 25μM iAs³⁺</td>
<td>13.75 ± 0.35</td>
<td>0.061</td>
</tr>
<tr>
<td>drp-1 + 100μM iAs³⁺</td>
<td>13.48 ± 0.33</td>
<td>0.022</td>
</tr>
</tbody>
</table>

P-values represent comparison (Mantel cox test) of control with each arsenite treatment group for each strain.
Table 5: 24h Arsenite LC<sub>50</sub> values.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (mM) at L4 stage (95% C.I.)</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (mM) at day 8 (95% C.I.)</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (mM) at day 12 (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>5.73 (2.93 – 8.52)</td>
<td>1.81 (1.06 – 2.57)</td>
<td>0.64 (0.45 – 0.84)</td>
</tr>
<tr>
<td>fzo-1</td>
<td>2.76 (2139 – 3376)</td>
<td>0.91 (0.66 – 1.16)</td>
<td>0.22 (0.10 – 0.35)</td>
</tr>
<tr>
<td>eat-3</td>
<td>2.97 (2.15 – 3.80)</td>
<td>0.38 (0.22 – 0.53)</td>
<td>0.12 (0.71 – 0.17)</td>
</tr>
<tr>
<td>drp-1</td>
<td>6.03 (2.42 – 9.64)</td>
<td>0.97 (0.69 – 1.26)</td>
<td>0.38 (0.23 – 0.54)</td>
</tr>
</tbody>
</table>
Table 6: Statistical analysis of Seahorse XF® and ATP results.

<table>
<thead>
<tr>
<th>Age / parameter</th>
<th>Arsenite</th>
<th>Strain</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4 Basal OCR</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>L4 ATP</td>
<td>P=0.012</td>
<td>P=0.0002</td>
<td>P=0.0055</td>
</tr>
<tr>
<td>L4 Maximal OCR</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P=0.36</td>
</tr>
<tr>
<td>L4 Spare capacity</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P=0.034</td>
</tr>
<tr>
<td>Day 8 Basal OCR</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Day 8 ATP</td>
<td>P=0.0002</td>
<td>P=0.0039</td>
<td>P=0.0096</td>
</tr>
<tr>
<td>Day 8 Maximal OCR</td>
<td>P=0.094</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Day 8 Spare capacity</td>
<td>P=0.17</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Day 12 Basal OCR</td>
<td>P=0.77</td>
<td>P=0.12</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Day 12 ATP</td>
<td>P=0.69</td>
<td>P&lt;0.0001</td>
<td>P=0.81</td>
</tr>
<tr>
<td>Day 12 Maximal OCR</td>
<td>P=0.33</td>
<td>P&lt;0.0001</td>
<td>P=0.003</td>
</tr>
<tr>
<td>Day 12 Spare capacity</td>
<td>P=0.14</td>
<td>P=0.015</td>
<td>P=0.012</td>
</tr>
</tbody>
</table>
Table 7: Summary of survival post-arsenite exposure analysis.

<table>
<thead>
<tr>
<th>Strain / treatment</th>
<th>Mean LS (Days ± SEM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 control</td>
<td>10.28 ± 0.79</td>
<td>NA</td>
</tr>
<tr>
<td>N2 + 100µM iAs$^{3+}$</td>
<td>9.74 ± 0.79</td>
<td>0.64</td>
</tr>
<tr>
<td>fzo-1 control</td>
<td>8.70 ± 0.84</td>
<td>NA</td>
</tr>
<tr>
<td>fzo-1 + 100µM iAs$^{3+}$</td>
<td>8.58 ± 0.74</td>
<td>0.80</td>
</tr>
<tr>
<td>eat-3 control</td>
<td>8.58 ± 0.76</td>
<td>NA</td>
</tr>
<tr>
<td>eat-3 + 100µM iAs$^{3+}$</td>
<td>7.90 ± 0.78</td>
<td>0.66</td>
</tr>
<tr>
<td>drp-1 control</td>
<td>9.92 ± 0.85</td>
<td>NA</td>
</tr>
<tr>
<td>drp-1 + 100µM iAs$^{3+}$</td>
<td>8.80 ± 0.73</td>
<td>0.16</td>
</tr>
</tbody>
</table>

P-values represent comparison (Mantel cox test) between control and arsenite (100µM) exposed nematodes for each strain.
Figure 24: Mitochondrial fusion- and fission-deficient nematodes are sensitive to arsenite.

(A) *fzo-1* and *eat-3*-deficient *C. elegans* are more sensitive to arsenite than wild-type (N2) nematodes in a 48h larval growth assay (two way ANOVA, arsenite, strain, interaction (p<0.0001 for all), N=471-1435). Larval growth was not measured for *fzo-1* or *eat-3* in the 800µM arsenite exposure group as significant lethality was observed. (B) *drp-1*-deficient nematodes were more susceptible to arsenite-induced reproductive toxicity than wild-type nematodes (two way ANOVA, arsenite, strain, interaction (p<0.0001 for all), N=11-19). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to N2 within each arsenite concentration. Bars ± SEM.
Figure 25: Mitochondrial fusion (fzo-1 & eat-3)-deficient C. elegans are sensitive to arsenite.

(A) Exposure to arsenite significantly reduced nematode development in a 48h larval growth assay (two-way ANOVA, arsenite, strain, interaction (p<0.0001 for all)). Growth measurements for fzo-1 and eat-3 could not be completed in the 800µM exposure group as significant mortality was observed. Data is represented as ToF, a surrogate for nematode length. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to the untreated control within each strain. N=471-475. Bars ± SEM.
Figure 26: Deficiencies in mitochondrial fusion sensitize C. elegans to Aflatoxin B₁

(A) Exposure to AfB₁ significantly reduced nematode growth in a 48h larval growth assay (two-way ANOVA, AfB₁, strain, interaction (p<0.0001 for all)). (B) fzo-1-, and eat-3-deficient nematodes were more sensitive to AfB₁ than wild-type (N2) nematodes (two-way ANOVA, AfB₁, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each AfB₁ concentration. N=353-590. Bars ± SEM.
Figure 27: Deficiencies in fission \((drp-1)\), and fusion \((fzo-1, eat-3)\) sensitize \(C.\) \textit{elegans to paraquat.}

(A) Exposure to paraquat significantly reduced nematode development in a 48h larval growth assay (two-way ANOVA, paraquat, strain, interaction \((p<0.0001\) for all)).

(B) \textit{fzo-1, eat-3,} and \textit{drp-1}-deficient nematodes were more sensitive to paraquat than wild-type (N2) nematodes (two-way ANOVA, paraquat, strain, interaction \((p<0.0001\) for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance \((p<0.05)\) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each paraquat concentration. \(N=442-780.\) Bars ± SEM.
Figure 28: Deficiencies in fusion (*fzo-1, eat-3*) and mitophagy (*pink-1*) sensitize *C. elegans* to rotenone.

(A) Exposure to rotenone significantly reduced nematode development in a 48h larval growth assay (two-way ANOVA, rotenone, strain, interaction (p<0.0001 for all)).

(B) *fzo-1, eat-3, pink-1*-deficient nematodes were more sensitive to rotenone than wild-type (N2) nematodes (two-way ANOVA, paraquat, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each rotenone concentration. N=367-575. Bars ± SEM.
Figure 29: Deficiencies in \textit{fzo-1}, \textit{eat-3}, and \textit{pink-1} mildly sensitize \textit{C. elegans} to the mitochondrial uncoupler 2,4-Dinitrophenol.

(A) Nematode growth was significantly reduced in all strains following a 48h larval exposure to 1000\(\mu\)M 2,4-DNP (two-way ANOVA, 2,4-DNP, strain, interaction (p<0.0001 for all)). (B) Deficiencies in fusion (\textit{fzo-1}, \textit{eat-3}) and mitophagy (\textit{pink-1}) mildly sensitized nematodes to 2,4-DNP, compared to wild-type (N2) nematodes (two-way ANOVA, 2,4-DNP, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain or (B) to N2 within each concentration of 2,4-DNP tested. N=310-379. Bars ± SEM.
Figure 30: Deficiencies in fzo-1 and eat-3 sensitize C. elegans to cisplatin.

Deficiencies in fusion (fzo-1, eat-3) sensitize nematodes to cisplatin-induced larval growth delay in 48h growth assay. Data is represented as percent control, and was generated from a (A) Copas Biosort based larval growth assay (two-way ANOVA, cisplatin, strain, interaction (p<0.0001 for all), N=121-200), and from (B) Fiji-based image analysis (two-way ANOVA, cisplatin, strain, interaction (p<0.0001 for all), N=47-66). Both methods of analyses gave similar results. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to N2 within each concentration of cisplatin tested. Bars ± SEM.
Figure 31: Deficiencies in fzo-1 and eat-3 mildly sensitize C. elegans to acetaldehyde.

(A) Exposure to acetaldehyde significantly reduced nematode development in a 48h larval growth assay (two-way ANOVA, acetaldehyde, strain, interaction (p<0.0001 for all)). (B) Fusion (fzo-1 & eat-3)-deficient C. elegans were mildly sensitive, while mitophagy (pink-1)-deficient nematodes were mildly resistant to acetaldehyde compared to wild-type (N2) nematodes (two way ANOVA, acetaldehyde, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each acetaldehyde concentration. N=864-1269. Bars ± SEM.
Figure 32: Deficiencies in fission, fusion, or mitophagy do not sensitize *C. elegans* to doxycycline.

(A) Exposure to doxycycline inhibited nematode growth and caused an L2/L3 larval arrest in all strains (two way ANOVA, doxycycline, strain, interaction (p<0.0001 for all)). (B) *pink-1*-deficient nematodes are mildly sensitive to doxycycline (two-way ANOVA, paraquat, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each doxycycline concentration. N=174-435. Bars ± SEM.
Figure 33: Deficiencies in fission, fusion, and mitophagy confer resistance to acrolein.

(A) Exposure to acrolein significantly reduced nematode development in a 48h larval growth assay (two-way ANOVA, acrolein, strain, interaction (p<0.0001 for all)).

(B) fzo-1-, eat-3-, drp-1-, pdr-1, and pink-1-deficient nematodes were resistant to acrolein compared to wild-type (N2) nematodes (two-way ANOVA, acrolein, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each acrolein concentration. N=477-705. Bars ± SEM.
Figure 34: *fzo-1*-deficient nematodes display mild resistance to cadmium.

(A) Exposure to cadmium reduced nematode growth in a 48h larval growth assay, causing complete L1 stage larval arrest in all strains at ≥150µM cadmium (two way ANOVA, cadmium, strain, interaction (p<0.0001 for all)). (B) *fzo-1*-deficient nematodes display mild resistance to low-dose (20-80µM) cadmium exposure (two way ANOVA, cadmium, strain, interaction (p<0.0001 for all)). Data is represented as (A) ToF, a surrogate for nematode length, and as (B) percent control. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to (A) the untreated control within each strain and (B) to N2 at each cadmium concentration. N=174-435. Bars ± SEM.
Figure 35: Arsenite reduces nematode lifespan in a strain-independent fashion.

Chronic, lifelong exposure to 100µM, but not to 25µM arsenite, significantly reduced nematode lifespan in (A) N2, (B) drp-1, (C) fzo-1, (D) eat-3 nematodes; however, (E) the overall reduction in mean lifespan between strains was not significantly different (two way ANOVA, arsenite (p<0.0001), strain (p=0.30) interaction (p=0.25)). Survival curves were analyzed via the Mantel cox test. Mean lifespan and p-values for statistical comparisons are shown in Table 4. N=75.
Figure 36: Deficiencies in fzo-1, eat-3 and drp-1 sensitize nematodes to arsenite-induced lethality throughout aging.

Note different x-axes in different panels. (A) L4 stage, (B) 8-, and (C) 12-day old fzo-1- and eat-3-deficient nematodes are hypersensitive to arsenite-induced lethality compared to wild-type nematodes, while only (B) 8-, and (C) 12-day old drp-1-deficient nematodes display sensitivity (2 way ANOVA (at each lifestage), main effects of strain, arsenite and interaction (p<0.0001 for all)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to N2 within each arsenite concentration. N=6-12. Bars±SEM.
Figure 37: Arsenite disrupts mitochondrial function in fusion-deficient nematodes.

Nematodes were exposure for 24h to non-lethal concentrations of arsenite, and then mitochondrial function (basal OCR, steady-state ATP, maximal OCR, and spare respiratory capacity) was assessed in (A-D) L4 stage, (E-H) 8-day old, and (I-L) 12-day old nematodes. P-values for statistical analysis are provided in Table 6. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to untreated control within each strain. N=11-13. Bars±SEM.
Figure 38: Arsenite does not alter mitochondrial morphology in wild-type nematodes.

24h Exposure to 100µM arsenite did not alter mitochondrial morphology in 8-day old wild-type nematodes.
Figure 39: Arsenite does not alter expression of fission or fusion genes in wild-type nematodes.

24h Exposure to 100µM arsenite did not alter expression of (A) *drp-1* (ANOVA (p=0.98)) or (B) *fzo-1* (ANOVA (p=0.36)) in wild-type nematodes. N=6. Bars±SEM.
Figure 40: *fzo-1; drp-1* double mutants display similar sensitivity to arsenite as *fzo-1* nematodes.

L4 stage *fzo-1; drp-1* double mutants display similar sensitivity to arsenite as *fzo-1* nematodes in a 24h lethality assay (one way ANOVA, strain, arsenite, interaction (p<0.0001 for all)). N=4. Bars±SEM.
Figure 41: Autophagy does not play a role in arsenite-induced mitochondrial dysfunction.

(A) A 12-hour pretreatment with 10mM 3-MA partially rescued arsenite-induced lethality in $fzo$-1-deficient nematodes (2-way ANOVA, arsenite ($p<0.0001$), strain ($p=0.033$), interaction ($p=0.033$)); however, 3-MA pre-treatment did not prevent arsenite from reducing basal OCR in $fzo$-1- or $eat$-3-deficient nematodes (2-way ANOVA, arsenite x 3-MA interaction ($p>0.05$ for each strain)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey’s HSD). N=10-11. Bars±SEM.
Figure 42: Arsenite does not induce expression of autophagy genes.

24h Exposure to 100µM arsenite did not alter expression of (A) *lgg*-1 (autophagy), (C) *dct*-1 (mitophagy), while expression of (B) *atg*-18 (autophagy) was reduced in *fzo*-1 (ANOVA, p=0.027). Asterisk denotes statistical significance (1-way ANOVA, p<0.05) for comparison between control and arsenite exposed nematodes within each strain. N=6. Bars±SEM.
Figure 43: Pretreatment with NAC, trolox, deferoxamine, or Mito-TEMPO fail to rescue arsenite-induced lethality.

(A) A 12 hour pretreatment with 100µM deferoxamine, 60µM NAC, 40µM trolox, or Mito-TEMPO failed to reduce arsenite-induced lethality in N2, fzo-1-, eat-3-, or drp-1-deficient nematodes. Following antioxidant pretreatment, each nematode strain was exposed to an equipotent concentration of arsenite. The effect of arsenite and each compound was analyzed via 2-way ANOVA for each strain (p>0.05 for all interaction terms). N=6. Bars±SEM.
Figure 44: Pretreatment with mitoQ partially rescues arsenite-induced lethality in eat-3- and drp-1-deficient nematodes.

(A) A 12 hour pretreatment with 5µM mitoQ partially rescues arsenite-induced lethality in eat-3- (2-way ANOVA, arsenite (p<0.0001), mitoQ (p=0.0018), interaction (p=0.0038)) and drp-1 (2-way ANOVA, arsenite (p<0.0001), mitoQ (p=0.0023), interaction (p=0.0073))-deficient nematodes compared to the TPP control. (B) However, mitoQ significantly altered basal OCR in control and arsenite treated fzo-1-, eat-3-, and drp-1-deficient nematodes (2-way ANOVAs, main effect of arsenite (p<0.05), but not interaction (p>0.05) term for each strain), thus obfuscating the results. N=6-11. Bars±SEM.
Figure 45: Arsenite-induces expression of superoxide dismutases.

(A) Arsenite induced cytosolic sod-1 expression in N2, fzo-1 and eat-3, (B) had no effect on mitochondrial sod-2 expression, (C) reduced mitochondrial sod-3 expression in N2 and fzo-1, and (D) induced extracellular sod-4 expression in fzo-1-deficient nematodes. Asterisk denotes statistical significance (1-way ANOVA, p<0.05) for comparison between control and arsenite exposed nematodes within each strain. N=5-6. Bars±SEM.
Figure 46: Arsenite induces the unfolded protein response.

24h Exposure to 100µM arsenite in 8-day old nematodes induced (A) cytosolic hsp-16.2 in all strains, (B) endoplasmic reticulum-specific hsp-4 in fzo-1, and (C) mitochondrial-specific hsp-6 in fzo-1 and eat-3, suggesting mitochondrial proteotoxicity may be playing a role in arsenite-induced toxicity in fusion-deficient nematodes. Asterisk denotes statistical significance (p<0.05) for comparison (one way ANOVA) to untreated control within each strain. N=5-6. Bars±SEM.
Figure 47: Arsenite inhibits PDH and IDH in fzo-1- and eat-3-deficient nematodes.

24h Exposure to 100µM arsenite in 8-day old nematodes inhibits (A) pyruvate dehydrogenase activity (two way ANOVA, strain (p=0.0022), arsenite (p<0.0001), interaction (p=0.041)) and (B) isocitrate dehydrogenase activity (two way ANOVA, strain, arsenite, interaction (p<0.0001 for all)) in fzo-1- and eat-3-deficient nematodes, suggesting a disruption of pyruvate metabolism and Krebs cycle activity underlie the observed mitochondrial dysfunction in fusion-deficient nematodes. Asterisk denotes statistical significance (p<0.05) for comparison (one way ANOVA) to untreated control within each strain. N=6-10. Bars±SEM.
Figure 48: Arsenite does not induce glycolysis.

(A) Exposure 50mM 2-deoxy-D-glucose (hexokinase inhibitor) did not significantly alter steady-state ATP levels in any strain following arsenite exposure (3 way ANOVA, p>0.05 for all effect and interaction terms, N=2). (B) Arsenite reduced gpd-3 expression in wild-type nematodes, while having no affect on expression in fzo-1, eat-3, or drp-1-deficient nematodes. Asterisk denotes statistical significance (p<0.05) for comparison (one way ANOVA) to untreated control within each strain. N=5-6. Bars±SEM.
Figure 49: Arsenite accumulates in *drp-1*-deficient nematodes.

(A) Increased arsenite was measured in crude lysate isolated from 8-day old *drp-1*-deficient nematodes exposed to 100µM arsenite for 24h (one way ANOVA, main effect of strain (p=0.046)); however, (B) arsenite did not differentially accumulate in isolated mitochondria (one way ANOVA, (p=0.82)). Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey HSD) comparison to N2. N=3. Bars±SEM.
Figure 50: ATP does not limit arsenite excretion via the ArsA ATPase.

(A) Arsenite does not induce asna-1 expression (1 way ANOVA (p>0.05 for all comparisons)). (B) Treatment with 500µM DCA increases steady-state ATP levels (2 way ANOVA, strain (p=0.0001), DCA (p=0.013), but not interaction (p=0.42)); however, (C) DCA treatment does not alter sensitivity to arsenite-induced lethality (3-way ANOVA, main effects of strain (p<0.0001), arsenite (p<0.0001), and strain*arsenite interaction (p<0.0001), but not DCA treatment (p>0.05)). N=4. Bars±SEM.
Figure 51: Arsenite does not induce expression of genes involved in mitochondrial biogenesis.

24h Exposure to 100µM arsenite in 8 day old nematodes did not alter (A) mitochondrial DNA polymerase gamma (polg-1) expression, or (B) hmg-5 expression in any strain, while a (C) small induction of mtDNA encoded nd-5 (ETC CI) was observed in eat-3, and decreased expression of ctb-1 (ETC CIII) was observed in wild-type nematodes. Asterisk denotes statistical significance (p<0.05) for comparison (one-way ANOVA) to untreated control within each strain. N=5-6. Bars±SEM.
Figure 52: Arsenite does not increase mitochondrial mass in wild-type nematodes.

8-day old nematodes expressing mitochondrial targeted GFP in body wall muscle cells were exposed to 100µM arsenite for 24h, and then GFP fluorescence was measured. GFP expression was normalized to nematode length (ToF). N=793-925.
Figure 53: Arsenite increases citrate synthase and ETC CI activity in wild-type nematodes.

24h exposure to 100µM arsenite in 8-day old nematodes increased (A) citrate synthase (two way ANOVA, strain (p=0.0029), arsenite (p=0.014), interaction (p=0.030), N=4), and (B) ETC CI activity in wild-type nematodes (two way ANOVA, strain (p=0.66), arsenite (p=0.045), interaction (p=0.030), N=4); however, the apparent trend in (C) increased mtDNA content in wild-type nematodes was not significant (two way ANOVA, strain (p<0.0001), arsenite (p=0.90), interaction (p=0.24), N=6). Asterisk denotes statistical significance (p<0.05) for comparison (Tukeys HSD) to untreated control within each strain. N=5-6. Bars±SEM.
Figure 54: Survival post-arsenite exposure.

Eight-day-old nematodes were exposed to 100µM arsenite for 24h and then survival was assessed. Arsenite exposure did not significantly affect the survival of (A) N2 (p=0.64), (B) fzo-1 (p=0.80), (C) eat-3 (p=0.66), or (D) drp-1-deficient nematodes (p=0.16). Survival curves were analyzed via Mantel cox test. Mean survival time (days ± SEM) is shown in Table 7. N=50.
Figure 55: Arsenite sensitizes nematodes to secondary stressors.

24h Exposure to 100µM arsenite sensitized nematodes to secondary exposure to the redox cycler (A) paraquat (two way ANOVA, strain (p=0.0007), arsenite (p<0.0001), but not interaction (p=0.62), n=6), while only sensitizing eat-3- and drp-1-deficient nematodes to (B) secondary thermal stress (37ºC) (survival curves analyzed via mantel cox test, N=40).
Figure 56: Enhancement of mitochondrial function is lost following 48h arsenite exposure.

(A) Basal OCR (one way ANOVA, P=0.38) and (B) steady-state ATP levels (one way ANOVA, P=0.99) were not affected by 48h exposure to 100µM arsenite in 8-day old wild-type nematodes. However, arsenite exposure reduced (C) maximal OCR (one way ANOVA, P=0.046), while the apparent trend in reduced (D) spare respiratory capacity (one way ANOVA, P=0.072) was not significant. Asterisk denotes statistical significance (p<0.05) for comparison (one way ANOVA) to untreated control. N=11. Bars±SEM.
4. Effects of reduced mitochondrial DNA content on secondary mitochondrial toxicant exposure in Caenorhabditis elegans

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4.1 Introduction

The mitochondrial genome (mtDNA) encodes 13 subunits of the electron transport chain (ETC), 22 tRNAs, and 2 rRNAs (283), thus critically linking mtDNA to energy production via oxidative phosphorylation (OXPHOS). Furthermore, the integrity of mtDNA is paramount for cellular and organismal health, which is demonstrated by the fact that mutations, deletions, and depletion of mtDNA cause severe respiratory chain disease in humans (284, 285). However, depending upon the age and energetic demands of a tissue, a cell can contain hundreds to thousands of copies of mtDNA (286), providing a cell with genetic redundancy that helps buffer against somatic and maternally-inherited mtDNA mutations. Thus, in the context of heteroplasmy (multiple mtDNA variants within a tissue), levels of pathogenic mutations must exceed a threshold, which generally ranges from 60-90% depending upon the severity of the mutation and the energy demands of the effected tissue, before overt pathology ensues (284, 285).

Another kind of mtDNA-related disease results from depletion of mtDNA, which can cause a severe group of disorders known as mtDNA depletion syndrome.
MDS (mitochondrial disease syndrome) is one of the most common childhood mitochondrial disorders (58), and is primarily caused by mutations in nuclear encoded genes that play a role in mitochondrial dNTP pool maintenance (TK2, DGUOK, RRM2B, TYMP, SUCLA2, SUCLG1) or mtDNA replication (POLG1, C10orf). This genetically heterogeneous group of disorders is characterized by a severe loss of mtDNA that results in impaired ATP production. The pathogenic threshold is dependent upon the energetic demands of the effected tissue, and severity of any concurrent mutations in the affected tissues or organs, which are most frequently brain, liver, kidney or muscle (56, 57). Mutations in the mitochondrial fusion gene (MFN2) (60, 61), and exposure to nucleoside reverse transcriptase inhibitors (NRTIs) (64, 65, 287), which are used to prevent the transmission of HIV from mother-to-child, can also causes severe tissue-specific reductions in mtDNA content.

Growing evidence has demonstrated that mitochondria and mtDNA are important targets of numerous drugs and environmental toxicants (66). The phospholipid rich mitochondrial double membrane attracts lipophilic genotoxicants (e.g. polycyclic aromatic hydrocarbons (PAHs), aflatoxins), while proton pumping gives the mitochondrial matrix a slight negative charge that attracts cationic compounds (Cd\(^{2+}\), Mn\(^{2+}\), Pb\(^{2+}\), EtBr\(^+\)) that can disrupt mitochondrial energy production through a number of routes. A lack of introns, protective histones, and some DNA repair pathways (e.g. nucleotide excision repair (NER)) contribute to mtDNA vulnerability to certain
genotoxicants, such as PAHs, which induce irreparable mtDNA damage due to a lack of NER (67, 68, 87, 288, 289). Although high mtDNA copy number provides genetic redundancy that may buffer against toxicant-induced mtDNA damage, little is known about how individuals with reduced mtDNA content will respond to environmental mitotoxicants.

Here, using the in vivo model organism Caenorhabditis elegans we tested the hypothesis that reduced mtDNA content (but not exceeding the pathogenic threshold) would increase vulnerability to secondary mitotoxicant exposure. As nematodes share highly conserved mitochondrial biology (112), energy metabolism pathways (290), and mtDNA (111) with humans, they represent an excellent in vivo model to test this hypothesis. Furthermore, availability of transgenic strains, such as the PE255 ATP-reporter strain (145, 291), allow for the rapid assessment of mitochondrial function in vivo. To test this hypothesis, we reduced mtDNA copy number 35-55% and then exposed nematodes to known and suspected mitochondrial toxicants (aflatoxin B₁ (AfB₁), arsenite, paraquat, rotenone, and ultraviolet C radiation (UVC)). Interestingly, the effects were not dramatic; we report mild exacerbation of mitochondrial dysfunction in the context of reduced mtDNA content in the context of some but not all exposures.
4.2 Methods

4.2.1 Strains and culture conditions

Luciferase-expressing, PE255 glp-4 (bn-2) nematodes were generously provided by Dr. Christina Lagido (University of Aberdeen, Aberdeen, UK). PE255 nematodes were maintained at the permissive temperature of 15°C until experiments were initiated, at which time they were shifted to the restrictive temperature of 25°C, which eliminates development of the nematode germline (292). Synchronous populations of larval stage one (L1) nematodes were generated via sodium hydroxide bleach treatment, followed by overnight incubation on an orbital shaker in complete K-medium (150µl 1M CaCl₂, 150µl 1M MgSO₄, 25µl 10mg/ml cholesterol, 50ml sterile K-medium (2.35g KCl, 3g NaCl, 1L ddH₂O)) at 20°C (148). Nematodes were then cultured on K-agar plates seeded with *Escherichia coli* OP50 as previously described (147, 149).

4.2.2 Ethidium bromide and toxicant exposure

Synchronous populations of L1 stage PE255 nematodes were cultured on OP50 seeded K-agar plates containing 0, 0.05 or 1.0µg/mL EtBr (dissolved in ddH₂O, Sigma-Aldrich, St. Louis, MO). EtBr exposures were continuously maintained for the duration of all experiments to maintain mtDNA knockdown.

**UVC exposure.** Synchronous populations of control and EtBr-exposed L4 stage nematodes were transferred onto OP50-free K-agar plates containing 0 or 1.0µg/ml EtBr, allowed to disperse, and then exposed to 0, 25, or 50 J/m² UVC using an ultra violet lamp
(UVLMS-38 EL Series 3UV Lamp, UVP, Upland, CA, USA) with peak emission at 254nm. Nematodes were then transferred back onto OP50 seeded control or EtBr plates for subsequent ATP determination.

**Chronic toxicant exposures.** Control and EtBr-exposed L4 nematodes were exposed to AfB1 (dissolved in 1% DMSO, Sigma-Aldrich) or arsenite (Ricca Chemical Company, dissolved in ddH2O) for 4 to 9 days using the spot dead method as previously described (293). Briefly, 1.5ml arsenite (0, 50, 250, 500µM) or AfB1 (0, 5, 25, 50µM in 1% DMSO) was added to the surface of peptone-free, control or EtBr (1µg/ml) K-agar plates and allowed to dry. K-agar plates were then seeded with 300µl 20X concentrated UVC-killed UvrA bacteria (UV-sensitive strain, due to lack of NER (224)). Nematodes were transferred daily to freshly prepared plates.

**Acute toxicant exposures.** Approximately 3,000 control and EtBr-exposed L4 stage nematodes were exposed to AfB1 (0, 25, 50µM in 1% DMSO), arsenite (0, 50, 100µM dissolved in ddH2O), methyl viologen dichloride (0, 0.5, 1.0mM paraquat hydrate dissolved in ddH2O, Sigma-Aldrich), or rotenone (0, 0.5, or 1.0µM dissolved in 1% DMSO, Sigma-Aldrich) in complete K-medium containing UVC-killed UvrA, and 0 or 1.0µg/ml EtBr for 48h on an orbital shaker at 25°C. Following toxicant exposure, nematodes were rinsed three times with 15ml k-medium to remove excess toxicant through dilution. Nematodes were then transferred back to control or EtBr containing plates and ATP levels were measured daily for 4 consecutive days.
4.2.3 Copy number determination

Nuclear (nucDNA) and mtDNA genome copy number was determined as previously described (231, 232). Briefly, 6 nematodes were added to 90µl proteinase K-containing lysis buffer using a platinum worm pick and frozen at -80°C. Samples were then thawed and lysed via a 1h incubation at 65°C. Crude nematode lysate was then used as template DNA for RT-PCR based determination of mtDNA and nucDNA copy number. Standard curves were used for absolute copy number determination. Copy number experiments were repeated 3 separate times.

4.2.4 Growth assay

An aliquot of nematodes grown on 0, 0.05, or 1.0µg/ml EtBr was collected every 2 days, for 12 consecutive days, and frozen at -20°C for size determination. On the day of analysis, samples were thawed and imaged at 10x magnification on a Zeiss Axioskop. Images were analyzed using NIS elements software (Nikon Inc., Melville, NY). Approximately 20 nematodes were measured for each exposure group within each time point from 2 independent experiments.

4.2.5 Determination of in vivo steady-state ATP levels

*In vivo* steady-state ATP levels were determined as previously described (145), and as visualized in (291). Briefly, 50 nematodes (suspended in K-medium) were loaded into each well of a white 96-well plate (four wells per treatment), and then GFP fluorescence was measured (emissions filter: 502nm; excitation filter: 485nm) using a
FLUOstar Optima microplate reader (BMG Labtech, Germany). 50µl of luminescence buffer (140mM Na$_2$PO$_4$, 30mM citric acid (pH 6.5), 1% DMSO, 0.05% Triton X-100, 100µM D-luciferin) was then injected into each well and nematode bioluminescence was measured 3 minutes later using a luminescence optic (BMG Labtech). As PE255 nematodes express a GFP::luciferase fusion protein, each well’s luminescence value was normalized to GFP fluorescence to correct for overall luciferase content and slight discrepancies in the number and size of nematodes loaded per well. All experiments were repeated at 3-5 separate times.

4.2.6 Lifespan

Twenty-five L1 stage PE255 nematodes were placed on OP50 seeded K-agar plates containing 0 or 1.0µg/ml EtBr at 25°C for the duration of their lives. Nematodes were scored daily, and judged dead when they failed to move in response to probing with a platinum worm pick. Lifespan experiments were repeated 2 separate times.

4.2.7 Seahorse XF$^e$ analysis

The fundamental parameters of the mitochondrial respiratory chain (basal oxygen consumption rate (OCR), maximal OCR, ATP-linked respiration, spare respiratory capacity (SRC), and proton leak) were measured following 4 and 8 days of exposure to EtBr using the Seahorse XF$^e$24 Bioanalyzer (Seahorse Bioscience, Massachusetts, USA) as previously described (228, 229). Briefly, 50 nematodes were loaded into each well of a Seahorse utility plate and 8 basal OCR readings were taken.
Nematodes were then exposed to either 25µM FCCP (mitochondrial uncoupler), 20µM DCCD (ATP synthase inhibitor), or 10mM sodium azide (cytochrome c oxidase inhibitor) and an additional 8, 16, or 4 oxygen consumption measurements were taken, respectively. SRC was calculated by subtracting a well’s basal OCR from its FCCP response, ATP-linked respiration was calculated by subtracting a well’s DCCD response from its basal OCR, while proton leak was calculated by subtracting a well’s azide response from its DCCD response. All experiments were repeated 3 separate times.

### 4.2.8 Gene expression

mRNA levels of two cytochrome P450s (CYP35B2, CYP33C6), three mitochondrial heat shock proteins (hsp-6, hsp-60a, hsp-60b), mitochondrial DNA polymerase (polg-1), and the nematode mitochondrial transcription factor A homolog (hmg-5) were measured using real-time PCR. mRNA was converted to cDNA using the Qiagen Omniscript Reverse Transcription kit. RT-PCR was performed using a 7300 Real Time PCR System (Applied Biosystems). The fold change of each gene was calculated by comparing the Ct of each gene to that of the housekeeping gene cdc-42 (237). RT-PCR primers for polg-1 and cdc-42 were based on the literature (153). Unpublished primers were as follows: CYP35B2 (For-5’-GTG GGC TGA AAT GCG AAG AT-3’, Rev-5’-CCA ACG GCA AGG TCA AAG AA-3’ (177bp amplicon, annealing temp 60ºC)); CYP33C6 (For-5’-GCT GCG GTT GTA TTC CT-3’, Rev-5’-GTC CAC CGT CTG AAG CAT TC-3’ (164bp amplicon, annealing temp 64ºC)); hsp-6 (For-5’-TCG TGT CAT CAA CGA GCC
AA-3', Rev-5'- AGC GAT GAT CTT ATC TCC AGC G-3' (76bp amplicon, annealing temp 56°C)); hsp-60a (For-5'-AGG CTC TTA CCA CTC TTG TTC T-3', Rev-5'- CTC CCG TCG CAA TTC CCA TA-3' (123bp amplicon, annealing temp 56°C)); hsp-60b (For-5'-CCA AGA AGG TCA CCA TCA CC-3', Rev-5'-TCT GTT TGA TCT CCA CGC CC-3' (64bp amplicon, annealing temp 56°C)); hmg-5 (For-5'-TGT CTG GAG CTG GAA TGG AA-3', Rev-5'-GCT TCT TCG CGT CTG TG-3' (108bp amplicon, annealing temp 60°C)). Samples were run in triplicate from two separate experiments.

4.2.9 Glycolysis assay

To detect changes in glycolysis, we exposed control, 100μM arsenite, EtBr, and EtBr and 100μM arsenite co-exposed nematodes to 2-deoxy-D-glucose (glycolysis inhibitor), and then measured the change in steady-state ATP as previously described (236, 245). Briefly, 50 nematodes were loaded into each well (4 wells per treatment) of a white 96-well plate and exposed to 50mM 2-DG for 4.5h at 25°C. Following 2-DG exposure steady-state ATP levels were determined as described above. The effect of 2-DG on each treatment group was then normalized to percent vehicle control (EPA H2O). The experiment was repeated four separate times.

4.2.10 Statistics

Copy number (mtDNA, nucDNA, and their ratio), gene expression, steady-state ATP, nematode growth, and Seahorse XF® data were initially analyzed with a one, two, or three way ANOVA, and when warranted post-hoc comparisons were made via
Tukey’s HSD. Lifespan data was analyzed via the non-parametric Mantel-Cox test. Statistics were performed using JMP v11.0 software (SAS Institute).

4.3 Results

As we were primarily interested in investigating the effects of mtDNA knockdown on somatic tissues, and development of the nematode germline during the L3/L4 transition is associated with large increases in mtDNA copy number (112, 151) which would confound the interpretation of our results, we used the germline-deficient, in vivo ATP reporter strain PE255 glp-4 for all experiments (145, 291). EtBr, a well-known inhibitor of mtDNA replication (294-296) that is frequently used to generate rho0 (mtDNA-deficient) cells, was used to reduce mtDNA copy number in nematodes.

4.3.1 Chronic, low dose EtBr exposure reduces mtDNA copy number without affecting nucDNA copy number, nematode lifespan, or larval development.

Chronic exposure to 1.0, but not 0.05µg/ml EtBr reduced mtDNA copy number 32-51% over the first 4 days of exposure (two way ANOVA, main effect of time (p<0.0001), not EtBr (p=0.07), and their interaction (p=0.013)) (Figure 57A), whereas EtBr had no effect on nucDNA copy number (two way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.09), or their interaction (p=0.36)) (Figure 57B), demonstrating EtBr’s ability to specifically inhibit mtDNA, but not nucDNA replication in C. elegans. Furthermore, a robust reduction (35-55%) in the ratio of mtDNA to nucDNA was observed over the first 8 days of EtBr exposure (two way ANOVA, main
effects of time (p<0.0001), EtBr (p<0.0001), and their interaction (p=0.0073) (Figure 57C).

Interestingly, the mtDNA:nDNA ratio returned to control levels by the twelfth day of exposure, which we postulate is due to reduced feeding and/or reduced uptake of EtBr through the nematode cuticle (a collagenous barrier known to limit toxicant uptake (163, 281, 297)) in aging nematodes, resulting in a loss of inhibition of mtDNA replication.

As chronic exposure to higher concentrations of EtBr (≥5.0µg/ml) has previously been reported to extend nematode lifespan through an induction of the mitochondrial unfolded protein response (UPR\textsuperscript{mt}) (151, 298), we tested whether lower concentrations of EtBr would elicit a similar effect. Chronic (48h) exposure to 1.0µg/ml EtBr elicited a mild, yet statistically significant induction of mitochondrial \textit{hsp-6} (one way ANOVA, p=0.0016) and \textit{hsp-60b} (p=0.0056), but not \textit{hsp-60a} (p=0.25) (Figure 58). However, lifelong exposure to 1.0µg/ml EtBr did not extend nematode lifespan (p=0.26) (Figure 59), suggesting that the low dose EtBr-induced UPR\textsuperscript{mt} is not robust enough to extend lifespan. Furthermore, 1.0µg/ml EtBr caused no larval growth delay (two way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.27) or their interaction (p=0.87)) (Figure 60), while 5.0µg/ml EtBr caused severe larval growth delay and L3 stage arrested (data not shown), a finding that has been previously reported (77). Collectively, these results demonstrate that in \textit{C. elegans}, chronic, low-dose EtBr exposure can effectively reduce mtDNA content, while having no observable adverse organismal level effects.
4.3.2 mtDNA knockdown causes mild disruption of the mitochondrial respiratory chain.

As the pathogenic threshold for mtDNA depletion syndrome is frequently reported to be around 65% (56, 57), we hypothesized that a 35-55% reduction in the whole worm mtDNA:nucDNA ratio would not cause major mitochondrial dysfunction. In agreement with this, only mild alterations in basal respiration (Figure 61A), ATP-linked respiration (Figure 61B), and proton leak (Figure 61D) were detected following chronic EtBr exposure (2 way ANOVA, main effects of Time (p<0.0001 for all) and EtBr (p<0.05 for all), but not their interaction (p>0.05 for all)), while 1.0µg/ml EtBr reduced spare respiratory capacity in 4 day old nematodes (2 way ANOVA, main effects of time (p=0.0009), and time*EtBr (p=0.0016), but not EtBr (p=0.93)) (Figure 61C), and increased FCCP-uncoupled maximal OCR in 8 day old nematodes (2 way ANOVA, main effects of time (p<0.0001), and time*EtBr (p=0.0074), but not EtBr (p=0.36)) (Figure 61A). Although increased maximal OCR in 8 day old nematodes exposed to EtBr was unexpected, compensatory changes in mtDNA and function have been observed following NRTI cessation (299), and we have previously observed increased mitochondrial DNA polymerase gamma expression in response to polymerase stalling, UVC-induced photodimers, which also result in large reductions in mtDNA copy number (153). As we observe an increase in mtDNA copy number from day 4 to day 12 (Figure 57A), which we hypothesis is due to reduced feeding in aging nematodes and thus reduced uptake of EtBr, we measured the effect of EtBr on DNA polymerase gamma (polg-1, the sole
replicative mitochondrial DNA polymerase), and \textit{hmg-5} (\textit{TFAM} homolog, required for mtDNA replication and transcription) expression to investigate a potential compensatory response that may explain EtBr-induced increases in maximal OCR. However, 1.0\mu\text{g/ml} EtBr did not affect \textit{polg-1} expression (Figure 62A), and only mildly increased \textit{hmg-5} expression (Figure 62B).

Although mild alterations in mitochondrial function were observed following chronic, low-dose EtBr exposure, no alterations in steady-state ATP levels were observed throughout the course of a 12 day EtBr exposure (2 way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.53), or their interaction (p=0.93)) (Figure 63), further demonstrating that the magnitude of EtBr-induced mtDNA knockdown (35-55\%) did not induce major mitochondrial dysfunction.

\textbf{4.3.3 Reduced mtDNA content sensitizes C. elegans to secondary exposure to some but not all mitotoxicants.}

Acute and/or chronic exposures to the known and suspected mitochondrial toxicants \textit{AfB\textsubscript{1}}, arsenite, paraquat, rotenone, and UVC were initiated after 48h of EtBr exposure in L4 stage nematodes, as this is when the most robust reduction in mtDNA (51\%) and the mtDNA:nucDNA ratio (55\%) was observed. With the exception of UVC (which does not penetrate the ozone layer), these toxicants are of human health concern. \textit{AfB\textsubscript{1}}, a foodborne mycotoxin and important carcinogen in the developing world, causes bulky DNA lesions when its epoxide metabolite reacts with DNA. We and others have found that \textit{AfB\textsubscript{1}} preferentially, and irreparably (due to a lack of NER) damages mtDNA.
Arsenite, a global drinking water contaminant associated with the development of skin, lung, and bladder cancer, is a well-known inhibitor of several Krebs cycle dehydrogenases (300), ETC complexes (301), and is also capable of causing metabolic shifts from OXPHOS to aerobic glycolysis, otherwise known as the Warburg effect (154, 245). Exposure to rotenone, a pesticide and prototypical mitotoxicant that acts by inhibiting complex I of the ETC leading to superoxide production, is associated with the development of Parkinson’s disease (88). Like rotenone, the herbicide paraquat is also associated with the development of Parkinson’s disease (88), but functions as a redox cycler that preferentially damages mtDNA (87); however, paraquat-induced mtDNA damage is oxidative and largely repaired by base excision repair. Alternatively, UVC causes equal amounts of nucDNA and mtDNA photodimers; however, photodimers are only repaired in the nuclear genome (302, 303), so that UVC can be used as a tool to rapidly induce irreparable mtDNA damage.

**Reduced mtDNA content does not sensitize nematodes to the effects of chronic arsenite or AfB₁ exposure on steady-state ATP levels.** Following mtDNA knockdown, nematodes were chronically (96h) exposed to arsenite or AfB₁. Neither arsenite (Figure 64) nor AfB₁ (Figure 65) differentially altered steady-state ATP levels between control and EtBr exposed nematodes (3 way ANOVA, p>0.05 for all). A non-significant graphical trend in reduced steady-state ATP levels in nematodes treated with EtBr was observed following 72 and 96h of AfB₁ exposure (Figure 65). Therefore, we chronically
exposed control and EtBr treated nematodes to AfB₁ for 9 days, starting at the L4 stage, to see if the observed effects on ATP would become more pronounced with a longer exposure. However, exposure to AfB₁ for 9 days at this concentration failed to significantly affect ATP levels in control or EtBr treated nematodes (Figure 66) (3 way ANOVA, p>0.05). P-values for all effects and interaction terms are shown in online Supplemental File 1.

As the nematode cuticle (a protective collagenous barrier) has been shown to limit toxicant uptake (163, 281, 297), and all chronic arsenite and AfB₁ exposures were performed on K-agar plates, we next performed liquid toxicant exposures, which has been shown to help facilitate toxicant uptake in nematodes (293).

**Reduced mtDNA content sensitizes nematodes to acute arsenite and UVC exposure, but not to AfB₁, paraquat or rotenone exposure, in liquid culture.** Following mtDNA knockdown nematodes were exposed to AfB₁, arsenite, paraquat and rotenone in liquid for 48h, and then steady-state ATP levels were determined every 24h for four days. Neither AfB₁ nor paraquat significantly altered steady-state ATP levels between control and EtBr exposed nematodes (Figure 67 & Figure 68) (3 way ANOVA, p>0.05 for both). Unfortunately, higher concentrations of AfB₁ could not be tested due to limited water solubility, while higher concentrations of paraquat (≥5mM) induced mortality.

Rotenone, a prototypical mitochondrial toxicant and ETC complex I inhibitor, reduced steady-state ATP levels 50% in both control and EtBr treated nematodes after a
48h rotenone exposure (Figure 69A). Unexpectedly, steady-state ATP levels remained reduced in control nematodes 24h after rotenone exposure, while ATP levels in nematodes treated with EtBr completely recovered by 24h, and then increased by 48h post-rotenone exposure (3 way ANOVA, rotenone*EtBr*Time interaction (p=0.0091)) (Figure 69A). This might suggest that nematodes with reduced mtDNA content recover more rapidly from rotenone exposure. An alternative hypothesis is that because rotenone is mainly metabolized via cytochrome P450s (304), and we have previously reported that high dose (5.0µg/ml) EtBr can induce CYP expression (153), the EtBr exposure protected against rotenone by increasing its metabolism. Therefore, we next tested whether the low doses (1.0µg/ml) of EtBr used in this study would also induce CYP expression. In agreement with our hypothesis, EtBr caused a very large (>180-fold) induction of CYP35B2 (one way ANOVA, p<0.001) (Figure 69B), but not CYP33C6 (p=0.27) (Figure 69C), two CYPs previously shown to play a role in xenobiotic metabolism in nematodes (305, 306). Unfortunately, it is not clear which of the many nematode CYP proteins is responsible for rotenone metabolism.

A persistent trend toward increased steady-state ATP levels was observed following arsenite exposure; however, reducing mtDNA copy number with EtBr prevented this trend (3-way ANOVA, EtBr*arsenite interaction (p=0.048)) (Figure 70A). As we have previously shown that similar concentrations of arsenite can induce glycolysis (245), we hypothesized that a persistent increase in glycolysis might be
responsible for the increased steady-state ATP levels. As previously reported, the glycolytic inhibitor 2-deoxy-D-glucose significantly reduced steady-state ATP levels in arsenite-treated nematodes immediately after arsenite exposure, demonstrating an induction of glycolysis (one way ANOVA, p=0.045) (Figure 70B). However, this effect was not observed in EtBr, or EtBr and arsenite co-treated nematodes, nor was it persistent (one way ANOVAs, p>0.05 for 24, 48, 72hrs).

Interestingly, a transient increase in steady-state ATP levels was observed 24h post UVC exposure in nematodes with reduced mtDNA content; however, steady-state ATP levels declined below control values 48 and 72h post-UVC, but then recovered by 96h (3 way ANOVA, time*arsenite*UVC interaction (p=0.049)) (Figure 71).

4.4 Discussion

Here, we have investigated how reduced mtDNA content affects sensitivity of the in vivo model organism C. elegans to secondary mitochondrial toxicant exposure, and report that reduced mtDNA content causes mild sensitization to certain environmental toxicants. Collectively, our results provide the first line of evidence that reduced mtDNA content may sensitize an organism to certain mitochondrial toxicants, while also demonstrating that this is not likely to be a universal outcome for all mitotoxicants.

4.4.1 Use of EtBr to reduce mtDNA genome copy number in germline-deficient nematodes.

Because development of the nematode germline around the L3/L4 transition causes large increases in whole-body mtDNA content that would complicate the
interpretation of results (151), we used germline-deficient glp-4 nematodes for our studies, which allows for the investigation of somatic cell effects of mtDNA knockdown. To reduce mtDNA, we used the DNA intercalating agent, EtBr, which has been used to generate rho^0 (mtDNA-deficient) cells for decades by preferentially inhibiting mtDNA replication over nucDNA replication (294-296). EtBr has previously been used to reduce mtDNA copy number in C. elegans; however, the timing and dose of EtBr exposure is crucial. High larval doses of EtBr (≥5.0µg/ml) induce larval growth arrest (77, 151), while the lower doses of EtBr (1.0µg/ml) used in the present study caused no growth delay (Figure 60). Interestingly, high doses of EtBr (≥5.0µg/ml) have also been shown extend nematode lifespan through induction of the mitochondrial unfolded protein response (UPR^mt) (151, 298). Because induction of the UPR^mt represent a protective response that could protect nematodes from secondary toxicant exposure, we utilized lower doses of EtBr to reduce mtDNA. Low dose EtBr (1.0µg/ml) mildly induced the expression of several genes (hsp-6, hsp60b, but not hsp-60a) known to play a role in the UPR^mt (Figure 58). However, chronic exposure to low dose EtBr did not extend nematode lifespan (Figure 59) suggesting only a mild induction of the UPR^mt.

Although chronic exposure to low dose EtBr reduced mtDNA 35-55% over the first 8 days of the nematode lifespan, the knockdown was lost by day 12 (Figure 57). The loss in knockdown may be explained by the nematode cuticle (a protective collagenous barrier), which has been shown to limit toxicant uptake, and is impermeable to ionic
compounds (163, 281, 297). However, nematode feeding also decreases with age (307, 308), which is expected to reduce intestinal uptake of EtBr, and contribute to the loss of mtDNA knockdown later in life. Interestingly, nematodes exposed to EtBr had increased maximal OCR after 8 days of exposure (Figure 61A). As compensatory changes in mtDNA and function have been reported following cessation of NRTI-induced mtDNA knockdown (299), we hypothesized that increased maximal OCR may be due to compensatory changes in mtDNA replication or transcription. To test this, we measured the expression of the mitochondrial DNA polymerase (polg-1), and the nematode TFAM homolog (hmg-5), which is required for mtDNA replication and transcription. However, chronic exposure to EtBr did not significantly affect polg-1 expression, and only mildly induced hmg-5 expression (Figure 62B). It is possible that the small induction of hmg-5 expression on day 8, which coincided with the increase in maximal OCR, facilitates a compensatory increase in mtDNA transcription. Additional experiments would be needed to test this possibility. Based on these findings, all secondary mitochondrial toxicant exposures were initiated in young adult (2 day old) nematodes, when the largest reduction of mtDNA was observed (55%), and all experiments were completed on or before day 8, when increased maximal OCR was observed.

4.4.2 C. elegans as a model for studying mtDNA depletion.

As mtDNA encodes 13 critical ETC subunits (283), depletion of mtDNA due to mutations in nuclear encoded genes that control mitochondrial mtDNA replication
(POLG1, C10orf) or the mitochondrial dNTP pool (TK2, DGUOK, RRM2B, TYMP, 
SUCLA2, SUCLG1) cause severe human respiratory chain disease. In agreement with the 
pathogenic threshold effect frequently described in other species, a 35-55% loss of 
mtDNA did not cause severe mitochondrial dysfunction in C. elegans. This is further 
supported by the fact that the NRTI, zidovudine, which reduced mtDNA content by 
greater than 80%, also reduced mitochondrial respiration in nematodes (309), while 
mutations in the sole mitochondrial polymerase, polg-1, resulted in a greater than 90% 
loss of mtDNA and altered mitochondrial morphology in C. elegans (152).

Although EtBr can be used to reduce mtDNA, and major mitochondrial 
dysfunction is not observed following a 35-55% reduction in mtDNA, there are 
limitations associated with the use of C. elegans to study mtDNA depletion. In humans, 
mtDNA depletion due to nuclear mutations typically only affects certain, energetically 
demanding tissues such as the brain, liver, kidney, or muscle (56, 57), and exposure to 
NRTIs has also been shown to deplete mtDNA in energetically demanding tissues (e.g. 
brain, heart, liver) in humans and primates (64, 310). Although nematodes lack many of 
the well-defined organs found in humans, they do have many well-defined tissues such 
as the cuticle, excretory system, gonad, hypodermis, intestine, muscles, neurons, and a 
pharynx (311). Some of these tissues, including the neurons, pharynx, and gonad, are 
more energetically demanding, and highly sensitive to certain toxicant exposures. For 
example, the nematode gonad is one of the most sensitive tissues to doxycycline- and
chloramphenicol-induced mitochondrial toxicity (238). Thus, it is possible that EtBr is preferentially depleting mtDNA in the more energetically demanding tissues of *C. elegans*; however, due to the small size of nematodes (adults are 1.0mm in length), it is difficult to isolate biochemically relevant amounts of purified tissue, making this hypothesis difficult to test.

### 4.4.3 Secondary toxicant exposures.

Given that high mtDNA copy number provides a cell with genetic redundancy and buffers against DNA damaging events, we hypothesized that reducing mtDNA copy number would sensitize nematodes to secondary mitochondrial toxicant exposure. Surprisingly, secondary toxicant exposure only mildly exacerbated mitochondrial dysfunction when initiated in the context of a 55% reduction in mtDNA. Several factors could explain this. First, many of the toxicants used in the present study have been shown to have tissue-specific effects in human and other mammalian models. For example, AfB₁ targets the liver (312, 313), while rotenone and paraquat target the central nervous system (314, 315), and arsenite targets many organs, such as the bladder, kidney, liver, lungs, and skin (316, 317). Given that more energetically demanding tissues are typically more sensitive to mtDNA depletion (56, 57), it is likely that the toxicants used in the present study are having tissue-specific effects or even exacerbating mitochondrial dysfunction in the context of reduced mtDNA copy number in a tissue-specific manner; however, it is also likely that these effects are being diluted at the
organismal level. This is supported by the fact that UVC, which indiscriminately damages mtDNA and nucDNA (irreparable in mtDNA) in all tissues of the transparent nematode (302, 303), reduced ATP levels in the context of reduced mtDNA, while AfB$_1$ and paraquat, both of which preferentially damage mtDNA (68, 87, 289), did not exacerbate mitochondrial dysfunction. Alternatively, the nematode cuticle may limit the uptake and thus toxicity of AfB$_1$, arsenite, paraquat, and rotenone, while not limiting UVC-induced damage. However, this seems unlikely as we have previously shown that the concentrations of AfB$_1$, paraquat and UVC used in the current study cause similar amounts of mtDNA damage (87, 303). Finally, EtBr can act synergistically with UV light (365nm) (318); however, we have previously shown that EtBr at this level does not exacerbate UVC (100-280nm)-induced mtDNA damage (153).

As previously mentioned, high concentrations of EtBr ($\geq$5 µg/ml) have been shown to induce the expression of numerous xenobiotic metabolizing genes, including myriad CYPs (153); however, little is known about how low-dose EtBr exposure will effect gene expression. Since steady-state ATP levels recovered faster in nematodes pretreated with EtBr than in nematodes not treated with EtBr following rotenone exposure, and rotenone is mainly metabolized via CYPs (304), low dose EtBr may be inducing the expression of CYPs, allowing EtBr-treated nematodes to metabolize and recover from rotenone exposure faster than nematodes not treated with EtBr. In agreement with this, CYP35B2 expression was induced by low doses of EtBr (Figure 69).
Although the precise CYPs that metabolize rotenone in mammals and nematodes remain unknown, both CYP35B2 and CYP33C6 have previously been shown to be inducible by high doses (5µg/ml) of EtBr (153), and are known to play a role in xenobiotic metabolism in nematodes (305, 306), providing a plausible explanation for why EtBr treated nematodes recovered more rapidly from rotenone exposure than control nematodes. However, it is important to note that induction of CYPs is not expected to be protective for all toxicant exposures, as paraquat is, in general, poorly metabolized (319), while CYPs metabolically activate AfB1 to its DNA damaging epoxide (although it is unclear if the necessary CYPs are being induced (CYP2A6, 2B6, 3A4); (320, 321)). Finally, nematodes lack arsenite methyl transferase (322), which plays a major role in arsenite metabolism and excretion in humans (323).

Interestingly, a trend in increased steady-state ATP levels was observed in nematodes exposed to arsenite, while reducing mtDNA copy number prevented this trend (Figure 70A). Although growing evidence suggests that the mitochondrion is an important target of arsenic toxicity (154, 245, 300, 301), low dose arsenic has also been linked to hormesis in which the induction of protective mechanisms is associated with therapeutic effects (278, 324). The reported horic effects of arsenic include: reduced risk for non-melanoma skin cancer in Denmark (277), increased growth advantage of cells in culture (23, 325), and lifespan extension in C. elegans (23). Furthermore, arsenite-induced lifespan extension in nematodes is accompanied by increased mitochondrial
respiration, and increased mitochondrial protein content (23). Although the concentrations of arsenite used in the present study are much higher (50-100µM vs. 100nM), the exposure duration is brief (48h vs. lifelong), which could result in increased mitochondrial function and steady-state ATP levels, while reduced mtDNA copy number may prevent this effect by limiting the number of mtDNA transcripts available for transcription and translation of ETC subunits. Alternatively, chronic arsenic exposure can induce metabolic shifts from OXPHOS to aerobic glycolysis (Warburg effect) in vitro, and in C. elegans (154, 245). Therefore, we hypothesized that arsenite-induced glycolysis persists after arsenite exposure, resulting in increased steady-state ATP levels. However, this does not appear to be the case, as sensitivity to ATP depletion following inhibition of glycolysis with 2-deoxy-D-glucose was not persistent in arsenite or EtBr and arsenite treated nematodes (Figure 70B).

Finally, given the long half-life of mtDNA in C. elegans (326), it is not surprising that we did not see reduced steady-state ATP levels in EtBr-treated nematodes exposed to UVC until 48h after UVC exposure. Although UVC induces irreparable photodimers in mtDNA, it is not surprising that steady-state ATP levels completely recovered by 96h-post UVC exposure, as we have previously shown that the processes of autophagy, and mitochondrial fission, fusion, and mitophagy play a role in the slow removal of irreparable mtDNA damage through the recycling of damaged genomes (77). It is also
possible that these protective mechanisms limit the adverse effects of the other mitochondrial toxicants tested.

4.5 Conclusions

Overall, our data suggests that individuals with reduced mtDNA content may be more susceptible to certain environmental toxicants. However, interpretation of our data is complicated by the fact that energetically demanding tissues tend to be more susceptible to mtDNA depletion, while many toxicants target specific tissues. It is likely, and an important area for future research, that adverse interactions between reduced mtDNA content and environmental toxicants will occur in a tissue specific manner.
Figure 57: Ethidium bromide reduces mtDNA copy number.

Chronic, lifelong exposure to EtBr reduced (A) mtDNA copy number (2 way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.073), and their interaction (p=0.013)), but not (B) nucDNA copy number (2 way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.089), or their interaction (p=0.36)), while also reducing the (C) mtDNA:nucDNA ratio (2 way ANOVA, main effect of time (p<0.0001), EtBr (p<0.0001), and their interaction (p=0.0073)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control within each time point. N=6-9. Bars ± SEM.
Figure 58: Ethidium bromide induces mitochondrial heat shock protein expression.

Chronic (48h) exposure to EtBr induced the expression of the mitochondrial heat shock proteins hsp-6 (one-way ANOVA, p=0.0016), hsp-60b (p=0.0056), but not hsp-60a (p=0.25) in L4 PE255 glp-4 nematodes. Asterisk denotes statistical significance compared (p<0.05) to control. N=6. Bars ± SEM.
Figure 59: Ethidium bromide does not alter nematode lifespan.

Time, on the x-axis, refers to both nematode age and the length of the EtBr exposure. Data was analyzed via the Mantel-Cox test (p=0.26). N=50.
Figure 60: Ethidium bromide does not alter nematode length.

Ethidium bromide does not alter nematode development (2-way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.27), or their interaction (p=0.87)). Time, on the x-axis, refers to both nematode age and the length of the EtBr exposure. N=20-59. Bars ± SEM.
Figure 61: Ethidium bromide induces mild alterations in mitochondrial respiration.

Reduced mtDNA content induced mild alterations in (A) basal respiration (2 way ANOVA, main effects of time (p<0.0001), and EtBr (p=0.0011), but not their interaction (p=0.45)), (B) ATP-Linked OCR (2 Way ANOVA, main effects of time (p<0.0001) and EtBr (p=0.036), but not their interaction (p=0.41)), and (D) proton leak (2 way ANOVA, main effects of time (p<0.0001), and EtBr (p<0.0001), but not their interaction (p=0.21)), while an increase in (A) FCCP-induced maximal OCR was observed after 8 days of EtBr exposure (2 way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.36), and their interaction (p=0.0074)), and reduced (C) spare capacity was observed after 4 days of EtBr exposure (2 way ANOVA, main effect of time (p=0.0009), but not EtBr (p=0.93), and their interaction (p=0.0016)). Time, on the x-axis, refers to both nematode age and the length of the EtBr exposure. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control within each time point. N=21-46. Bars ± SEM.
Chronic, lifelong exposure to EtBr does not affect (A) polg-1 expression (two way ANOVA, main effect of time ($p=0.0048$), but not EtBr or their interaction ($p>0.05$ for both)), but does induce the expression of (B) hmg-5 (two way ANOVA, main effects of time ($p<0.0001$) and EtBr ($p=0.041$), but not their interaction ($p=0.26$)), the nematode homolog of TFAM, which plays a role in mtDNA replication and transcription. Time, on the x-axis, refers to both nematode age and the length of the EtBr exposure. Data is normalized to fold change over the day 2 control samples. N=6. Bars ± SEM.
Chronic, lifelong exposure to EtBr does not affect steady-state ATP levels throughout the nematode lifespan (2 way ANOVA, main effect of time (p<0.0001), but not EtBr (p=0.53), or their interaction (p=0.93)). Time, on the x-axis, refers to both nematode age and the length of the EtBr exposure. Relative luminescence is a surrogate measure of \textit{in vivo} steady-state ATP levels. N=2. Bars ± SEM.

Figure 63: Ethidium bromide does not alter steady-state ATP levels.
Figure 64: Reduced mtDNA content does not sensitize nematodes to chronic arsenite exposure.

Panel A shows the effect of chronic (96h) agar arsenite exposure on relative luminescence, a surrogate for *in vivo* steady-state ATP levels, while panel B shows ATP normalized to percent untreated control. Data was assessed via 3 way ANOVA (p-values shown in Supplemental File 1). N=3. Bars±SEM.
Figure 65: Reduced mtDNA content does not sensitize nematodes to chronic (4 day) AfB₁ exposure.

Panel A shows the effect of chronic (96h) agar AfB₁ exposure on relative luminescence, a surrogate for in vivo steady-state ATP levels, while panel B shows ATP normalized to percent untreated control. Data was assessed via 3 way ANOVA (p-values shown in Supplemental File 1). N=3. Bars±SEM.
Figure 66: Reduced mtDNA content does not sensitize nematodes to chronic (9 day) AfB1 exposure.

Panel A shows the effect of chronic (9 day) agar AfB1 exposure on relative luminescence, a surrogate for in vivo steady-state ATP levels, while Panel B shows ATP normalized to percent untreated control. Data was assessed via 3 way ANOVA (p-values shown in Supplemental File 1). N=2. Bars±SEM.
Figure 67: Reduced mtDNA content does not sensitize nematodes to acute (48h) AFB1 exposure.

Panel A shows the effect of acute (48h) liquid AFB1 exposure on relative luminescence, a surrogate for in vivo steady-state ATP levels, while Panel B shows ATP normalized to percent untreated control. Data was assessed via 3 way ANOVA (p-values shown in Supplemental File 1). N=4. Bars±SEM.
Figure 68: Reduced mtDNA content does not sensitize nematodes to acute (48h) paraquat exposure.

Panel A shows the effect of acute (48h) liquid paraquat exposure on relative luminescence, a surrogate for in vivo steady-state ATP levels, while Panel B shows ATP normalized to percent untreated control. Data was assessed via 3 way ANOVA (p-values shown in Supplemental File 1). N=4. Bars±SEM.
Figure 69: Nematodes with reduced mtDNA content recover from rotenone faster than control nematodes.

(A) Steady-state ATP levels were reduced 50% in control and EtBr treated nematodes following a 48h liquid rotenone exposure; however, steady-state ATP levels recovered to control levels faster in nematodes co-exposed to EtBr and rotenone (3 way ANOVA, EtBr*Time*Rotenone interaction (p=0.0091), N=3). 48h exposure to EtBr induced the expression of (B) CYP35B2 (one way ANOVA, p<0.0001, N=6), but not (C) CYP33C6 (one way ANOVA, p=0.27, N=6). P-values for all effect and interaction terms are shown in Supplemental File 1. Relative luminescence values are shown in Supplemental Figure 8. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control within each time point. Bars±SEM.
Figure 70: Reduced mtDNA content sensitizes *C. elegans* to arsenite.

(A) A persistent trend in increased steady-state ATP levels was observed following a 48h liquid exposure to arsenite, while reducing mtDNA content with EtBr prevented the observed trend (3 way ANOVA, EtBr*arsenite interaction (p=0.048)). P-values for all effect and interaction terms are shown in Supplemental File 1. Relative luminescence values are shown in Supplemental Figure 9. (B) Inhibition of glycolysis with 2-deoxy-D-glucose significantly reduces steady-state ATP levels in nematodes treated with 100µM arsenite immediately after (T=0) arsenite exposure demonstrating the induction of glycolysis (one way ANOVA, p=0.045). However, this effect was not observed in nematodes treated with 1.0µg/ml EtBr or co-treated with EtBr and arsenite, nor is the induction of glycolysis persistent (p>0.05 for one way ANOVAs 24, 48, 72h post arsenite). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3-5. Bars±SEM.
Figure 71: Reduced mtDNA content sensitizes C. elegans to UVC.

Steady-state ATP levels were reduced 48-72h post-exposure to a single dose of 25 or 50 j/m² UVC in nematodes with reduced mtDNA content (3 way ANOVA, EtBr*Time*UVC interaction (p=0.049)). P-values for all effect and interaction terms are shown in Supplemental File 1. Relative luminescence values are shown in Supplemental Figure 10. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control within each time point. N=5. Bars±SEM.
5. Conclusions

5.1 Summary

The overall goal of this work was to broaden our understanding of how genetic
deficiencies in mitochondrial homeostasis processes can sensitize an organism to known
and suspected mitochondrial toxicants. Much of our limited understanding of
mitochondrial gene-environment interactions comes from the pharmaceutical industry,
which is not surprising, as toxicity testing on pharmaceuticals is more thorough than
testing on chemicals used in, or produced as a result of, industrial processes. Point
mutations in mitochondrial 12s rRNA that render the rRNA more “bacteria-like” in
structure can result in ototoxicity following treatment with aminoglycoside antibiotics
due to inhibition of mitochondrial protein synthesis (91). In another example, inborn
errors in metabolism can sensitize individuals to valproic acid-induced liver
hepatotoxicity (89, 90). However, very little is known about how genetic deficiencies in
mitochondrial function can sensitize individuals to environmental toxicants, many of
which are known to target mitochondria (66). Therefore, the first hypothesis addressed
in this work was that deficiencies in mitochondrial fission, fusion, and mitophagy would
sensitize the model organism Caenorhabditis elegans to known and suspected
mitochondrial toxicants.

Deficiencies in mitochondrial fusion genes (fzo-1, eat-3) sensitized nematodes to a
variety of toxicants, including AFB1, arsenite, cisplatin, paraquat, rotenone, and 2,4-DNP,
while deficiencies in fission (drp-1) and mitophagy (pdr-1, pink-1) only resulted in mild sensitization. These results highlight the importance of mitochondrial fusion in limiting mitochondrial toxicity. Given what is known about the processes of fission, fusion, and mitophagy, these results fit well with the literature. Highly fragmented mitochondria, such as those found in fusion-deficient nematodes, are typically considered less metabolically active, while elongated mitochondria are frequently more metabolically active (36, 37). Furthermore, fusion promotes mitochondrial content mixing, which can buffer against damage, and this protective response is lost in fusion-deficient nematodes (38, 205).

Further investigation of the molecular mechanisms underlying arsenite toxicity revealed a disruption of mitochondrial function in arsenite exposed fusion-deficient nematodes. Interestingly, pyruvate and isocitrate dehydrogenase activity was reduced, suggesting a disruption of pyruvate metabolism and Krebs cycle activity underlie the mitochondrial dysfunction. Arsenite is well-known inhibitor of these enzymes in multiple model systems (222, 245, 327, 328). As we have previously shown that longer duration arsenite exposures can disrupt pyruvate metabolism in germline-deficient nematodes (Appendix B, (245)), these results suggest that the mechanisms by which arsenite induces mitochondrial dysfunction are conserved between wild-type and fusion-deficient nematodes; however, fusion-deficient nematodes are more sensitive to
this mechanism of toxicity, as shorter duration arsenite exposures induce mitochondrial
dysfunction.

In chapter 4 of this work, we tested the hypothesis that reduced mtDNA content
would sensitize nematodes to secondary mitochondrial toxicant exposure. Using a
chronic, low-dose ethidium bromide exposure we were able to reduced mtDNA content
35-55% over the first eight days of the nematode lifespan. However, reducing mtDNA
content only mildly sensitized nematodes to certain toxicants (UVC, arsenite), and only
under certain exposure conditions. These results suggest reduced mtDNA content may
sensitize individuals to certain toxicant exposures; however, further work is needed to
clarify the extent of sensitivity, and contexts in which it is important. For example, a
more robust depletion of mtDNA (>55%) may result in a more dramatic sensitization to
toxicants. Finally, and as discussed further below, MDS typically occurs in a tissue
specific manner; thus, re-testing this hypothesis in a mammalian model may reveal more
dramatic tissue-specific interactions between reduced mtDNA content and toxicants that
were lost in our nematode model.

5.2 Broader Impacts

This work makes important contributions to the fields of environmental
toxicology, mitochondrial biology, and human mitochondrial disease.

Although many mitochondrial disease are rare, collectively, 1 in 4,000 suffer
from mitochondrial disease (129); thus, mitochondrial disease represents a large public
health burden. Interestingly, the age of onset, and severity of clinical manifestations of many mitochondrial diseases vary, even for patients with identical mutations, suggesting a role for the environment in the development, progression, and exacerbation of certain mitochondrial disorders. Indeed, mounting evidence supports this. For example, mtDNA mutations that cause Leber hereditary optic neuropathy have an incomplete penetrance (50% in males, 10% in females); however, heavy smoking and alcohol consumption can increase penetrance to 93% in males and 33% in females (329); valproic acid can cause fatal hepatotoxicity in individuals with inborn errors in metabolism (89, 90); gentamycin can induce ototoxicity in individuals with mutations in mtDNA encoded 12s rRNA (91); ethambutol has been reported to exacerbate neuropathies in a CMT2A patient and induce optic atrophy in patients with OPA1 mutations (100, 101, 330). However, experimental evidence confirming the precise molecular interactions is lacking.

Data presented in this dissertation builds upon our understanding of mitochondrial gene-environment interactions. In particular, mitochondrial fusion appears to be an especially important biological response in limiting mitochondrial toxicity. This is supported by growing evidence from other literature demonstrating the importance of mitochondrial dynamics in response to stressors. Exposure to manganese, 6-hydroxydopamine, or glutamate can induce mitochondrial fission and toxicity; however, inhibition of fission prevents toxicity (261-263). Furthermore, OPA1-deficient
rat cortical neurons have been reported to be sensitive to rotenone (331), while inner membrane fusion-deficient *C. elegans* and *D. melanogaster* have been reported to be sensitive to paraquat (250, 332), and *drp-1*-deficient *C. elegans* have been reported to be mildly sensitive to paraquat (333). Here we confirm and expand upon these findings, demonstrating that both *fzo-1* - and *eat-3*-deficient nematodes are sensitive to paraquat and rotenone, while also confirming a mild paraquat sensitivity in *drp-1*-deficient nematodes. As paraquat is an amphiphilic redox cycler that can accumulate in mitochondria and generate ROS, while rotenone is an ETC CI inhibitor that can generate the superoxide anion, these results collectively suggest that individuals harboring mutations in mitochondrial fusion genes may be especially susceptible to toxicants that induce mitochondrial ROS production.

Previously, we have shown that the processes of mitochondrial fission, fusion, and mitophagy are required for the slow removal of bulky, irreparable, UVC-induced photodimers in the mitochondrial genome (76, 77). However, only deficiencies in mitochondrial fusion genes (*fzo-1*, *eat-3*) sensitize nematodes to larval growth arrest following UVC-induced mtDNA damage, suggesting deficiencies in fusion may sensitize individuals to irreparable mtDNA damage. Here we have shown that deficiencies in fusion genes also sensitize nematodes to AFB1 and cisplatin. Interestingly, both AFB1 and cisplatin are also capable of inducing irreparable mtDNA damage (87, 259), which is suggests that individuals with mitochondrial fusion-deficiencies may also
be especially sensitive to toxicants capable of inducing irreparable mtDNA damage. This is of great environmental importance, because many common contaminants cause such damage, including polycyclic aromatic hydrocarbons that are ubiquitous air pollutants.

5.3 Future Directions

Although *C. elegans* is a powerful *in vivo* model for studying mitochondrial biology, toxicity, and dysfunction, it does have limitations. For example, nematodes’ small size limits researchers’ ability to investigate tissue-specific mitochondrial dysfunction. Instead, mitochondrial function is typically assessed in whole nematodes or whole nematode lysates. This is an important limitation, as many toxicants have tissue-specific effects, while mitochondrial disease also frequently occurs only in specific, high-energy demand, tissues. For example, AfB1 targets the liver (312, 313), chronic exposure to paraquat and rotenone can target the central nervous system (314, 315), while MDS typically affects energetically demanding tissues (Brain, liver, muscle) (56, 57) and mutations in *OPA1* and *MFN2* mainly affect neuronal function (43, 208). Thus, it is likely that many tissue specific effects are lost or diluted in our *C. elegans* model. Further investigation of mitochondrial gene-environment interactions in a more complex model may reveal more robust, tissue-specific effects.

Finally, our results suggest that deficiencies in mitochondrial fusion may result in sensitization to certain classes of drugs and / or toxicants (discussed in section 5.2),
including those that generate mitochondrial ROS and those that induce irreparable mtDNA damage. However, further mechanistic studies are required to confirm this.
Appendix A – Effects of chronic, low-dose arsenite on mitochondrial function in fission- and fusion-deficient nematodes.

One of the original goals of this dissertation was to investigate the effects of chronic, low-dose (25µM) arsenite on mitochondrial function in fission- and fusion-deficient nematodes. As nematodes are a terrestrial species, long-term liquid culture can stress nematodes, confounding experimental results. Thus, chronic arsenite exposures were performing by directly adding arsenite to sterile agar prior to its solidification and seeding with the nematode food source.

A.1 Methods

Basal OCR and steady-state ATP levels were assessed as described in Appendices C and B, respectively, while nematode length was assessed using NIS Elements software (Nikon Inc., Melville, NY) as described in Chapter 4.

A.1.1 Arsenite exposure

K-agar was sterilized using an autoclave (as described in Appendices C & D), allowed to cool to 55°C, and then arsenite was added to a final concentration of 25µM. Agar was then seeded with UV-inactivated UVRA bacteria (as described in Chapters 3-5). Age-synchronized L1 stage nematodes were then pipetted onto control or arsenite containing plates and allowed to develop at 20°C. Basal OCR and ATP levels were assessed at the fourth larval stage and at eight days of age.
A.1.2 Secondary rotenone exposure

Following eight days of arsenite exposure, ten N2, *fzo-1*, *eat-3*, or *drp-1* nematodes were added to control or rotenone (0.5, 1.0, 2.5, 3.5, 5.0, 10, 20µM) containing K-agar plates using a platinum worm pick. Nematodes were then incubated at 20°C for 24h and scored as dead if they failed to move in response to repeated probing with a worm pick.

**A.2 Results and Discussion**

Chronic, low-dose (25µM) arsenite did not significantly affect larval development in any strain (Figure 1), nor did life-long exposure to 25µM arsenite significantly alter the lifespan of any strain (Figure X; Chapter 3). Nevertheless, chronic low-dose arsenite reduced basal OCR and steady-state ATP levels in L4 stage *eat-3* and *drp-1*-deficient nematodes, while 8 days of exposure reduced basal OCR in *fzo-1* and *drp-1*-deficient nematodes, and interestingly increasing steady-state ATP levels in *drp-1* nematodes (Figure 2 A-B). Finally, eight day exposure to arsenite sensitized both *drp-1* and *eat-3*-deficient nematodes to secondary rotenone exposure (Figure 3).

Collectively, these results demonstrate that chronic, low-dose arsenite can exacerbate mitochondrial dysfunction in fission- and fusion-deficient nematode strains. However, these results contrast, to some extent, with our findings in Chapter 3, where we demonstrate that deficiencies in mitochondrial fusion consistently sensitize nematodes to acute (24h) arsenite toxicity, while sensitivity to acute arsenite toxicity is more limited in fission-deficient nematodes. Here, in Appendix A, we provided
evidence that fission-deficient nematodes are sensitive to chronic, low-dose arsenite exposure. These results may be reflective of the biology of mitochondrial fission and fusion. For example, fusion promotes both elongation of the mitochondrial network (as seen in \textit{drp-1} nematodes) and functional complementation (38, 205). Thus, hyperfusion and functional complementation may provide \textit{drp-1}-deficient nematodes with protection from acute arsenite exposures; however, this rapid (fusion can occur in minutes), protective response is lost in \textit{fzo-1} and \textit{eat-3} nematodes. On the other hand, elongated mitochondria also tend to be recalcitrant to autophagy, and inhibition of fission results in the accumulation mitochondrial damage throughout the aging process in multiple species (41, 77). Thus, it is possible that chronic arsenite exposure promotes the accumulation of mitochondrial damage throughout the aging process in \textit{drp-1}-deficient nematodes due to impaired autophagosomal degradation, while damaged mitochondria in fusion-deficient nematodes can still be disposed of via mitophagy.
Figure 72: Chronic exposure to 25µM arsenite does not affect larval development.

Larval growth was assessed at the fourth larval stage by measuring nematode length. Chronic agar exposure to 25µM arsenite did not affect the development of any strain (2-way ANOVA, strain (p=0.58), arsenite (p=0.41), interaction (p=0.80)). N=20-29. Bars ± SEM.
Figure 73: Chronic arsenite exposure mildly exacerbates mitochondrial dysfunction in fzo-1-, eat-3-, and drp-1-deficient nematodes.

(A) Chronic exposure to 25µM arsenite reduced basal OCR in L4 stage and 8-day old drp-1-deficient nematodes. Alternatively, basal OCR was only reduced in L4 stage eat-3 and 8-day old fzo-1 nematodes (3-way ANOVA, strain*arsenite*age interaction (p=0.049)). (B) Chronic arsenite exposure reduced ATP levels in L4 eat-3- and drp-1-deficient nematodes, while increasing ATP levels in drp-1 nematodes after 8-days of exposure (3-way ANOVA, strain*arsenite*age interaction (p=0.044), N=10-16). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) between control and arsenite treated nematodes for each strain. Bars ± SEM.
Figure 74: Arsenite sensitizes *eat*-3 and *drp*-1 nematodes to secondary rotenone exposure.

Eight day agar exposure to 25µM arsenite sensitized *eat*-3- and *drp*-1-deficient nematodes to rotenone induced lethality compared to age-matched controls (3-way ANOVA, strain*arsenite*rotenone interaction (p=0.0011)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) between control and arsenite treated nematodes within each strain for a given rotenone concentration. N=4-6. Bars ± SEM.
Appendix B – Arsenite uncouples mitochondrial respiration and induces a Warburg-like effect in Caenorhabditis elegans.

This chapter was published under the same title in *Toxicological Sciences* in 2016 (PMID: 27794142). The authors are Anthony L. Luz, Tewodros R. Godebo, Dhaval P. Bhatt, Olga R. Ilkayeva, Laura L. Maurer, Matthew D. Hirschey, Joel N. Meyer.

**B.1 Introduction**

Over 140 million people worldwide consume arsenic contaminated drinking water that exceeds the World Health Organization’s limit of 10ppb (216), with some of the highest concentrations of arsenic occurring in ground water in Bangladesh and West Bengal (334). In this region of the world 35-77 million people are exposed to drinking water arsenic concentrations that range from less than 10ppb to over 4ppm, and as many as one in five deaths are attributed to arsenic exposure (334, 335). Although the United States complies with the WHO’s maximum contaminant level for municipal water supplies, private wells are rarely monitored, leaving millions of individuals at risk for chronic arsenic exposure. Lower levels of arsenic exposure may also be problematic. In New Hampshire, where 40% of residents own private wells, 10% of which exceed the 10ppb limit (336), exposure has been associated with the development of squamous cell carcinomas (217) and bladder cancer (218), suggesting that private wells need to be monitored or that the current contaminant limit is not stringent enough.
In addition to skin (219) and bladder (220) cancer, arsenic is also associated with kidney (337), liver (338), and lung (339) cancer, and has been implicated in metabolic syndrome (222, 223), and other metabolism-related pathologies (221, 340, 341). However, despite decades of research, arsenic’s precise mechanism(s) of inducing disease remains poorly understood. Reactive oxygen species (ROS) production (248), DNA damage (342), altered DNA methylation (260), and enzyme inhibition (251) are all thought to play a role. Growing evidence indicates that mitochondria are an important cellular target of arsenic toxicity. Arsenite can enter mitochondria via aquaglyceroporins, where it can bind and inhibit numerous enzymes involved in energy production, including pyruvate, succinate, isocitrate, and α-ketoglutarate dehydrogenases (252-254), as well as complexes II and IV of the electron transport chain (ETC) (301), suggesting arsenic-induced mitochondrial dysfunction may play a role in arsenic-related pathologies.

Interestingly, chronic, low-dose (75ppb) arsenite exposure was recently shown to induce the Warburg effect, which is defined as a shift from mitochondrial oxidative phosphorylation (OXPHOS) to aerobic glycolysis and is a hallmark in the development of many cancers, in several pulmonary epithelial cell lines (154). Importantly, the observed glycolytic shift was accompanied by reduced Krebs cycle activity, increased aneuploidy, and a loss of anchorage dependent growth, all of which were dependent upon stabilization of the transcription factor, hypoxia inducible factor-1 alpha (HIF-1A) (343), further implicating disruption of mitochondrial energy metabolism as an
important mechanism in arsenic carcinogenesis. The potential for environmental exposures to contribute to carcinogenesis via altered mitochondrial metabolism has recently been reviewed, but there are as yet few examples of such a mechanism (344).

However, the aforementioned studies were performed in vitro, and because mitochondrial function is highly dependent upon cellular context and intercellular signals (135), many of which are lost in vitro, it is important to test these results in vivo. The model organism Caenorhabditis elegans represents an excellent in vivo model for studying arsenic-induced mitochondrial dysfunction, as mitochondrial biology (112) and core metabolic pathways (290) are well conserved with humans. Furthermore, transgenic strains such as the PE255 ATP reporter strain (145), as well as strains with mutations in mitochondrial genes can be used to rapidly assess the in vivo mitochondrial effects of arsenic. Here, utilizing C. elegans, we demonstrate an in vivo induction of glycolysis, which is accompanied by severe mitochondrial dysfunction (altered pyruvate metabolism, increased proton leak, and reduced ATP, ATP-linked respiration, and spare respiratory capacity) following exposure to arsenite. Our results both confirm in vivo outcomes observed previously in vitro, and demonstrate novel mitochondrial outcomes, further expanding our knowledge of the mechanisms underlying arsenite-induced metabolic dysfunction.
**B.2 Methods**

**B.2.1 C. elegans strains and culture conditions**

Age-synchronized populations of L1 (larval stage 1) nematodes were obtained via sodium hydroxide bleach treatment as previously described (148), followed by an overnight incubation in complete K-medium (150µl 1M CaCl$_2$, 150µl 1M MgSO$_4$, 25µl 10mg/ml cholesterol, 50ml sterile K-medium (2.35g KCl, 3g NaCl, 1L ddH$_2$O)) on an orbital shaker at 20°C. Synchronized *C. elegans* were then cultured on K-agar plates seeded with *Escherichia coli* OP50 as previously described (147, 149), while arsenite exposed nematodes were fed UVC-inactivated *E. coli* (UvrA-deficient strain, which lacks nucleotide excision repair and thus cannot repair UVC-induced DNA damage). Inactivated UvrA was generated via exposure to 1000J/m$^2$ UVC using an ultra violet lamp (UVLMS-38 EL Series 3UV Lamp, UVP, Upland, CA, USA) with peak emission at 254nm as described (345).

Wild type (N2 Bristol), ETC complex I (MQ1333 *nuo-6* (*qm*200; outcrossed x 6)), complex II (TK22 *mev-1* (*kn*1; outcrossed x 5)), complex III (MQ887 *isp-1* (*qm*150; outcrossed x 3), MQ989 *isp-1*(*qm*150; outcrossed x 3);*ctlb-1* (*qm*189)), complex V (LB127 *atp-2* (*ua*2; outcrossed x 6)), pyruvate kinase (VC1265 *pyk-1* (*ok*1754; outcrossed x 0)), lactate dehydrogenase (VC1767 *ldh-1* (*gk*3142; outcrossed x 0)), uncoupling protein 4 (CY121 *ucp-4* (*ok*195; outcrossed x 5)), beclin-1 (VC517 *bec-1* (*ok*691; outcrossed x 1)), VC893 *atg-18* (*gk*378; outcrossed x 1), hypoxia inducible factor-1 (ZG31 *hif-1a* (*ia*4;
outcrossed x 9), ZG596 hif-1a (ia7; outcrossed x 4), and superoxide dismutase (sod-2 (gk257); sod-3 (tm760), sod-1 (tm776); sod-4 (gk101); sod-5 (tm1146)) mutants were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota), and maintained at 20°C. The germline-deficient strain JK1107 glp-1 (q224; outcrossed >1) was also purchased from the CGC, while the transgenic, luciferase-expressing PE255 glp-4 (bn2) strain was generously provided by Dr. Christina Lagido, University of Aberdeen (Aberdeen, UK). Germline deficient strains were maintained at the permissive temperature of 15°C until the time of the experiment when they were shifted to the restrictive temperature of 25°C. All nematode strains will henceforth be referred to by their gene name.

B.2.2 Arsenite exposure

For all experiments, approximately 5,000 young adult PE255 glp-4 or glp-1 nematodes were exposed to 0, 50, 250, or 500µM sodium arsenite (Ricca Chemical Company, dissolved in ddH2O) in complete K-medium containing UVC-killed UvrA (killed UvrA was added in a 1:5 ratio of bacterial culture resuspended in complete K medium to exposure medium) for 12, 24, or 48h (12 and 24h exposures were only performed for metabolomics analysis) while shaking at 25°C. Following all arsenite exposures, nematodes were rinsed four times with K-medium to remove excess bacteria and arsenite, and then placed on an orbital shaker for 20 minutes at 25°C to allow bacteria to clear from the nematode gut. Nematodes were then rinsed a final time with
unbuffered reconstituted moderately hard water (EPA H$_2$O, 60mg MgSO$_4$·7H$_2$O, 60mg CaSO$_4$·2H$_2$O, 4mg KCl per 1L ddH$_2$O) (150) prior to assessing the mitochondrial endpoints outlined below.

**B.2.3 Metabolic inhibition assay**

To rapidly assess the effects of arsenite on mitochondrial function, we developed a novel assay that permits *in vivo* assessment of the relative degree of chemical-induced inhibition of different metabolic processes that contribute to maintenance of ATP levels (236). Changes in ATP levels following short-term incubation with well-known inhibitors of mitochondrial energy metabolism (optimized conditions presented in Table 8) were measured in arsenite-exposed nematodes. Following 48h of arsenite exposure, nematodes were resuspended in unbuffered EPA water to a final concentration of 1.0 ± 0.2 nematodes/µl. Approximately 50 nematodes per sample were pipetted into 96-well plates using pipette tips rinsed in 0.1% Triton X-100 to prevent worm loss due to sticking. Four technical replicates (i.e. four wells) of each treatment were run per experiment. Inhibitors (listed in Table 8) were added to achieve 1X final concentration, and the remaining volume was adjusted to 100µl with unbuffered EPA water. Appropriate EPA water, DMSO controls, and four blank (unbuffered EPA H$_2$O) wells were included in each 96-well plate.

Following inhibitor exposure, as outlined in Table 8, steady state ATP levels were measured as previously described (145, 291). Briefly, GFP fluorescence was
measured (emissions filter: 502nm; excitation filter: 485nm) using a FLUOstar Optima plate reader (BMG Labtech, Offenburg, Germany). Luminescence buffer (140mM Na₂PO₄, 30mM citric acid (pH 6.5), 1% DMSO, 0.05% Triton X-100, 100µM D-luciferin) was injected into each well and luminescence was measured three minutes later using a luminescence optic (BMG Labtech). Luminescence values were then normalized to GFP expression. The average effect of each inhibitor on ATP levels in arsenite exposed nematodes was then normalized to either the EPA water or DMSO control within each treatment group, and expressed as percent change of control. All experiments were repeated at least three separate times.

**B.2.4 Metabolomics analysis**

Nematodes (glp-1) were exposed to arsenite for 12, 24 or 48h as outlined above, and then frozen for metabolomics analysis. Briefly, *C. elegans* samples were rinsed once with ice cold PBS, resuspended in 300µl ddH₂O containing 0.6% formic acid, flash frozen in liquid nitrogen, and then stored at -80°C until metabolite extraction.

To extract metabolites, all samples were thawed on ice and then sonicated on ice, using five 30 second on-off pulses at 20% power (Biologics, Inc., VA, USA). An aliquot was saved for total protein analysis via bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL). Remaining sample was mixed with acetonitrile (1:1 ratio), vortexed, and divided in a 1:3 ratio for amino acid and acyl carnitine, and organic acid quantification. Amino acids and acylcarnitines were analyzed using MS/MS as previously described.
and organic acids were quantified using GC/MS as described in (348). Raw data were normalized to protein, and then converted to log₂-fold change relative to untreated nematodes.

**B.2.5 Seahorse analysis**

Using a Seahorse XF²4 Bioanalyzer (Seahorse Bioscience, Massachusetts, USA), we measured the fundamental parameters of the mitochondrial respiratory chain: basal oxygen consumption rate (OCR), ATP-linked respiration, maximal OCR, spare respiratory capacity (SRC), and proton leak, as previously described (228, 229) (Appendix C). Briefly, nematodes were suspended in unbuffered EPA water to a final concentration of 1.0 ± 0.2 nematodes/µl. 50 nematodes were then pipetted into each well of a 24-well utility plate (six or seven wells for each treatment, leaving two wells as blanks), and the final well volume was brought to 525µl with unbuffered EPA water. An aliquot of nematodes was then collected and stored at -80°C for total protein determination. Following an initial eight basal OCR measurements, nematodes were exposed to either 25µM FCCP (mitochondrial uncoupler), 20µM DCCD (ATP synthase inhibitor) or 10mM sodium azide (cytochrome c oxidase inhibitor), followed by an additional eight, fourteen, or four OCR measurements, respectively, for each inhibitor. SRC was calculated by subtracting a well’s average basal OCR from its FCCP-induced maximal OCR. ATP-linked respiration was calculated by subtracting a well’s DCCD response from its basal OCR, whereas proton leak was calculated by subtracting a well’s
response to sodium azide from the well’s response to DCCD. OCR measurements were normalized to total protein.

### B.2.6 Steady-state ATP levels

Steady state ATP levels were measured in *glp-1* nematodes as previously described (349). Briefly, nematodes were flash frozen in K-medium and stored at -80°C until time of analysis. On the day of analysis, 10% trichloroacetic acid was added to each frozen sample, and samples were allowed to thaw on ice. 0.5mm Zirconia beads were then added to each sample, and samples were then homogenized using a bullet blender (Next Advance, Averill Park, NY), run at maximum speed for three 45s pulses at 4°C. Samples were then neutralized via the addition of 1.33mM KHCO₃ and Sigma water (St. Louis, MO, USA). Aliquots of each sample were then saved on ice for total protein determination. The remainder of each sample was then transferred to a new micro-centrifuge tube, vacuum centrifuged at 4°C for 10 minutes to remove air bubbles, and finally centrifuged at 14,000rpm for eight minutes at 4°C to pellet protein. Samples were then diluted 1:10 and 1:50 in sigma water for ATP determination via the Molecular Probes ATP Determination Kit (Invitrogen/ Life Technologies, Carlsbad, CA). Luminescence was measured using a FLUOstar Optima plate reader equipped with a luminescence optic. Calculated ATP concentrations were normalized to total protein.
B.2.7 Mitochondrial and nuclear DNA copy number determination

Mitochondrial (mtDNA) and nuclear (nucDNA) DNA copy number were determined as previously described (232). Briefly, six nematodes were added to lysis buffer containing proteinase K using a platinum worm pick, and frozen at -80°C. Thawed samples were then lysed via a 1h incubation at 65°C. Crude worm lysate was then used as template DNA for real-time PCR determination of copy number. Standard curves were used for absolute determination of mtDNA and nucDNA copy number. Three samples of each treatment were collected per experiment. Each experiment was repeated three separate times.

B.2.8 Isolation of mitochondria for ICP-MS analysis

Following arsenite exposure, nematodes were rinsed two times with ice cold MSM buffer (20.04g mannitol, 11.98g sucrose, 523.5mg MOPS, 0.5L milliQ H₂O, pH 7.4), transferred to a glass homogenizer and homogenized for three minutes. Homogenate was then transferred to an ultracentrifuge tube and spun at 300 x G for 10 minutes at 4°C. The supernatant was then transferred to a new tube, while the pellet was re-homogenized, spun down, and supernatants from the two homogenization steps were pooled. The pooled supernatant was then spun at 7000 x G for 10 minutes at 4°C to yield the mitochondrial pellet. The supernatant was then discarded, and the pellet was resuspended in MSM buffer, and re-pelleted two additional times to rinse the mitochondrial fraction, which was then resuspended in 600µl MSM buffer. An aliquot
was then saved for protein determination, while the remaining sample was used for arsenic analysis. Briefly, samples were poured into acid-cleaned 15mL teflon vials. Concentrated HNO₃ acid (1.5mL, 15N), 0.5 mL of ddH₂O, and 5 drops of 30% H₂O₂ were added to the samples, and the vials were sealed. The samples were then heated progressively on a hotplate from 50 to 80°C for 12h, while regularly being degassed (after 30 minutes cooling) until the digestion step was completed. The digested samples were analyzed via a VG Plasmaquad 3 inductively coupled plasma–mass spectrometer.

**B.2.9 Larval growth assay**

Larval growth assays were performed as previously described (138). Briefly, 25 L1 stage N2, *nuo-6, mev-1, isp-1, isp-1;ctb-1, atp-2, pyk-1, ldh-1, ucp-4*, or *bec-1* nematodes were loaded into a 96-well plate using a COPAS Biosort (Union Biometrica Inc., Somerville, MA) (four wells per strain per treatment). The final volume of each well was then brought to 100µl with complete EPA water (500µl 10mg/ml cholesterol (dissolved in ethanol), 96mg NaHCO₃, 60mg MgSO₄·7H₂O, 60mg CaSO₄·2H₂O, 4mg KCl per liter ddH₂O), UVC-killed UvrA, and sodium arsenite to a final concentration of 0, 200, 400, 600, or 800µM arsenite. Nematodes were then allowed to develop for 48h at 20°C. Time of flight ((ToF), a surrogate for nematode length) was then measured using the COPAS Biosort as previously described (138). All experiments were repeated three separate times. As all strains do not grow at the same rate, growth data was normalized to percent growth of control (within strain) prior to statistical analysis.
B.2.10 Lethality assay

Ten L4 stage N2, atg-18, bec-1, hif-1a (ia4 and ia7), sod-1;4;5, or sod-2;3 nematodes were loaded into a 96-well plate using a platinum worm pick (two wells per strain per treatment). The final volume of each well was then brought to 100µl with complete K-medium, UVC-killed UvrA, and sodium arsenite to a final concentration of 0, 1, 2, 3, 4, 5mM arsenite. 24h later, nematodes were scored as dead if they failed to move in response to repeated probing with a platinum worm pick. All experiments were repeated three separate times.

B.2.11 PDH activity assay

PDH activity was measured in isolated mitochondria following 48h of arsenite exposure using a Pyruvate Dehydrogenase Activity Colorimetric Assay Kit (BioVision, Cat. # K679). Mitochondria were isolated as described above, and resuspended in BioVision’s PDH Assay Buffer. PDH activity was then measured following manufacturer’s instructions. All experiments were repeated three separate times.

B.2.12 Mitochondrial membrane potential

Mitochondrial membrane potential was measured using the dual-emission potential-sensitive dye JC-1 (Sigma-Aldrich), which fluoresces red with high membrane potential, and green at lower membrane potential. Briefly, 3µM JC-1 (dissolved in 1% DMSO) was added to control and arsenite-exposed nematodes for the final 12h of arsenite exposure. Following the exposure, nematodes were rinsed 3-4 times, and
allowed to clear their guts. Nematodes were then placed on a 10% agar pad with 100mM levamisole (paralytic used for imaging) and imaged on a Zeiss 510 upright confocal microscope using a 63x water-immersion objective (Light Microscopy Core Facility, Duke University). To standardize the mitochondrial population assayed, the posterior pharyngeal bulb was chosen as the region of interest. Red and green pixel intensities within this anatomically identifiable area were measured using ImageJ. A 1h exposure to 50µM FCCP (2% DMSO) was used as a positive control. Four nematodes were imaged per experiment, which was repeated two separate times.

**B.2.13 Statistics**

All statistical analysis were performed using JMP v11.0 software (SAS Institute). All data was initially assessed using a one or two way ANOVA, and when warranted post-hoc analysis was performed using Tukey’s Honest Significant Difference test.

**B.3 Results**

Trivalent arsenite was used in all experiments because it is one of the predominant arsenicals found in groundwater and is generally more toxic than pentavalent arsenate (350). As we were primarily interested in investigating the mitochondrial effects of arsenite in somatic tissues, and development of the nematode germline is associated with dramatic changes in mitochondrial biology (including increased OXPHOS and mtDNA copy number) that could confound our results we used germline-deficient PE255 glp-4 or glp-1 nematodes for all experiments.
B.3.1 Arsenite increases glycolysis and reduces the function of ETC complexes II and V

To rapidly test for altered mitochondrial function in response to arsenite exposure, we developed a novel assay that permits *in vivo* assessment of the relative degree of chemical-induced inhibition of different metabolic processes (e.g., OXPHOS and glycolysis) that contribute to the maintenance of steady-state ATP levels (236). Transgenic, luciferase-expressing, PE255 *glp-4* nematodes were exposed to 0, 50, 250, or 500µM arsenite. Although these concentrations of arsenite fall below the previously reported nematode 24h LC₅₀ value of 1.3mM (256), some mortality was observed in PE255 *glp-4* nematodes exposed to the highest concentration of arsenite (500µM, qualitative observation, A. Luz). Therefore, this concentration was not used with PE255 *glp-4* nematodes. Transgenic PE255 nematodes express ATP-powered firefly luciferase, which allows for the rapid, *in vivo* determination of steady-state ATP levels (145, 291); thus, after 48h of arsenite exposure we measured real-time changes in ATP levels after short-term (1, 3 or 4.5h) exposure to various inhibitors of the ETC, glycolysis, or the Krebs cycle (Table 8). The principal that underlies this assay is that differential depletion of ATP indicates altered function (defined here as maintenance of steady-state *in vivo* ATP levels) at the site of inhibition in response to arsenite exposure. For example, if inhibition of complex I normally results in a decrease in ATP levels of 70%, but after arsenite exposure the same inhibition results in a decrease of only 35%, this suggests that
arsenite exposure is reducing complex I function, and thus its overall contribution to steady-state ATP levels.

Steady-state ATP levels were reduced in a dose-dependent manner following a 48h arsenite exposure (one way ANOVA, p<0.0001) (Figure 75A). To assess the relative contributions of different metabolic processes that contribute to the maintenance of ATP levels, we normalized ATP levels after each inhibitor to the appropriate EPA H2O (2-DG, DCA), 1% DMSO (rotenone, TTFA, antimycin A, DCCD) or 2% DMSO (FCCP) controls. 2% DMSO (1h exposure) had no significant effect on ATP levels (one way ANOVA, p=0.25); however, 1% DMSO (3h exposure) did cause a small, but significant reduction (~16%) in ATP levels in nematodes exposed to 250µM arsenite (one way ANOVA, p=0.009) (Figure 75B-C).

Arsenite exposure had no effect on ETC complex III (one way ANOVA, p=0.75), IV (one way ANOVA, p=0.99) or pyruvate dehydrogenase kinase (one way ANOVA, p=0.20) activities (Figure 76). The apparent trend towards reduced complex I activity (i.e. reduced sensitivity to ATP depletion following rotenone exposure) observed following arsenite exposure was not statistically significant (one way ANOVA, p=0.069) (Figure 75D). Interestingly, reduced complex II activity was observed in nematodes exposed to both 50 and 250µM arsenite (one way ANOVA, p<0.0001; p=0.030 and p<0.0001, respectively, for pairwise comparisons to control) (Figure 75E), while only 250µM arsenite reduced ATP synthase activity (one way ANOVA, p<0.0001; P<0.0001 for
pairwise comparison to control) (Figure 75F). Furthermore, 50, but not 250µM arsenite reduced nematode sensitivity to ATP depletion following exposure to the mitochondrial uncoupler FCCP (one way ANOVA, p=0.038; p=0.045) and p=0.090, respectively, for pair-wise comparisons to control) (Figure 75G), while inhibition of glycolysis with 2-DG reduced ATP levels in nematodes exposed to both 50µM and 250µM arsenite (one way ANOVA, p=0.017; p=0.049 and p=0.017, respectively, for pairwise comparisons to control) (Figure 75H).

Although reduced ATP depletion following TTFA, DCCD, and FCCP exposure in arsenite treated nematodes may be due to compensatory increases in ATP production via alternative routes such as glycolysis, we do not expect this to be the case. If compensatory increases in glycolysis were maintaining ATP levels, then all ETC inhibitors should elicit smaller reductions in ATP in the context of arsenite exposure. Collectively, these results indicate that arsenite disrupted OXPHOS and induced aerobic glycolysis, suggesting the induction of a Warburg-like effect.

**B.3.2 Arsenite disrupts pyruvate metabolism**

To further characterize the metabolic details and time course of the observed shift from OXPHOS to aerobic glycolysis in transgenic PE255 *glp*-4 nematodes, we quantified metabolites important to energy production following 12, 24, and 48h of 0, 50, or 500µM arsenite exposure using non-transgenic, germ line-deficient *glp*-1 nematodes. Interestingly, *glp*-1 nematodes exposed to 500µM arsenite appeared quite healthy and no
mortality was observed (qualitative observation, A. Luz); thus, as with the previous experiment, the highest concentration of arsenite (500µM) tested was chosen based on the fact that it caused no mortality. Mean metabolite concentrations, as well as log₂ fold change, standard error, and p-values for all statistical analyses are listed in online Supplemental File 1.

Exposure to 500µM arsenite resulted in the accumulation of pyruvate after 12 (one way ANOVA, p=0.0088), 24 (one way ANOVA, p=0.0011), and 48h (one way ANOVA, p=0.0002) of arsenite exposure (Figure 77A), whereas lactate only accumulated detectably following 48h of exposure (one way ANOVA, p=0.048) (Figure 77B). No Krebs cycle intermediates were significantly altered in response to arsenite exposure, though trends in reduced citrate (one way ANOVA, p=0.056), and increased malate (one way ANOVA, p=0.085) were observed following 24 and 48h of arsenite exposure, respectively (online Supplemental File 1), suggesting mild disruption of Krebs cycle activity. Because these results suggest that arsenite disrupted pyruvate metabolism, we measured pyruvate dehydrogenase (PDH) activity after 48h of arsenite exposure, and found a dose-dependent reduction in PDH activity (one way ANOVA, p=0.0016) (Figure 77C).

Because amino acid catabolism can fuel energy metabolism, we quantified amino acid levels following arsenite exposure. Glycine, the only amino acid altered after 12h of exposure, was elevated (one way ANOVA, p=0.014), whereas aspartate/asparagine (one
way ANOVA, p=0.044), glycine (one way ANOVA, p=0.035), and valine (one way ANOVA, p=0.045) were all elevated following 24h of exposure to 500µM arsenite (Supplemental File 1). Interestingly, 11 amino acids (Ala, Asx, Cit, Glx, Gly, His, Leu/Ile, Orn, Ser, Tyr, Val) were elevated following 48h of exposure to 50 and/or 500µM arsenite (one way ANOVA’s for each amino acid, p<0.05, as listed in Supplemental File 1), while non-statistically significant trends in the same direction (increased) were also observed for arginine (one way ANOVA, p=0.070), methionine (one way ANOVA, p=0.061), and phenylalanine (one way ANOVA, p=0.054) (Figure 78). This amino acid profile is consistent with increased autophagy and/or proteasomal degradation in response to arsenite exposure, as previously reported in vitro (246, 247). Using a genetic approach, we carried out additional experiments to test for a functional role for autophagy in tolerating arsenic toxicity. Autophagy protects against arsenic toxicity: bec-1-deficient nematodes are mildly, yet statistically significantly sensitive to arsenite exposure (two way ANOVA, main effects of strain (p<0.0001), arsenite (p<0.0001), and their interaction (p=0.013)) in a 48h larval growth assay (Figure 79) and in a 24h lethality assay (Figure 80), while atg-18-deficient nematodes are highly sensitive to arsenite ((two way ANOVA, main effects of strain, arsenite and their interaction (p<0.0001 for all) Figure 80)).

Because these results suggest an induction of autophagy following arsenite exposure, we hypothesized that increased autophagy might lead to increased mitochondrial turnover, and thus possibly altered mtDNA copy number (assuming no
compensatory change in biogenesis). However, no significant changes in mtDNA (one way ANOVA, \( p=0.15 \)), nucDNA (one way ANOVA, \( p=0.99 \)), or their ratio (one way ANOVA, \( p=0.87 \)) were observed following 48h of arsenite exposure (Figure 81).

Interestingly, a two-fold increase in alanine was observed in nematodes exposed to both 50 and 500\( \mu \)M arsenite (Figure 78), which could result from increased transamination of pyruvate to alanine, further suggesting a disruption of pyruvate metabolism. Likewise, large increases in citrulline and ornithine were observed (Figure 78), suggesting disruption of the urea cycle; however, functional urea cycle enzymes are yet to be identified in \textit{C. elegans} (351).

Acetyl-CoA can also enter the Krebs cycle, fueling energy metabolism, through \( \beta \)-oxidation of fatty acids. Thus, we quantified acylcarnitine levels, a biomarker of acyl-CoA species (352), following arsenite exposure. Levels of several short (C3, C5, C5-OH/C3-DC, C6, C6-DC/C8-OH, C8) and medium chain (C14:1, C14:2) acylcarnitines were mildly increased following exposure to arsenite (Supplemental File 1). However, arsenite exposure did not alter any long chain acylcarnitines, suggesting arsenite has minimal effect on fatty acid oxidation.

**B.3.3 Arsenite reduces ATP content, ATP-linked respiration, and spare respiratory capacity, while increasing proton leak**

Because the majority of alterations in metabolites (i.e. amino acids, pyruvate, and lactate) were observed following 48h of arsenite exposure, we characterized the functional consequences of these alterations on mitochondrial respiration (basal OCR,
spare respiratory capacity (SRC), ATP-linked respiration, and proton leak) using the Seahorse XF Bioanalyzer after 48h of arsenite exposure in *glp-1* nematodes.

Unexpectedly, basal OCR was not significantly altered by arsenite exposure (Figure 82A) (one-way ANOVA, \( p=0.061 \)). However, ATP levels were significantly reduced by 50, but not 500\( \mu \)M arsenite (Figure 82B) (One-way ANOVA, \( p=0.042 \); \( p=0.032 \) for pairwise comparisons to control). Interestingly, a dose dependent reduction in ATP-linked respiration (Figure 82C) (one way ANOVA, \( p<0.0001 \); \( p<0.0001 \) for both pairwise comparisons to control) and increase in proton leak were observed following arsenite exposure (Figure 82E) (one way ANOVA, \( p<0.0001 \); \( p<0.0001 \) for both pairwise comparisons to control), while both 50 and 500\( \mu \)M arsenite severely reduced spare respiratory capacity (Figure 82D) (one way ANOVA, \( p<0.0001 \); \( p<0.0001 \) and \( p=0.0001 \), respectively, for pairwise comparisons to control). Arsenite altered mitochondrial respiration in a similar manner (e.g. increased proton leak, and reduced ATP-linked respiration and spare capacity) in PE255 *glp-4* nematodes (Figure 83), demonstrating that arsenite induced similar alterations in mitochondrial respiration in both genetic backgrounds.

To further test the role of proton leak, which is defined as the transport of protons across the inner mitochondrial membrane independent of ATP synthase activity, we measured mitochondrial membrane potential, which should be reduced by mitochondrial uncoupling, using the potential-sensitive dye JC-1. As expected, arsenite
reduced mitochondrial membrane potential (one way ANOVA, p=0.0023) (Figure 84). Next, we tested whether nematodes deficient in mitochondrial (sod-2;3) and non-mitochondrial (sod-1;4;5) superoxide dismutase would be sensitive to arsenite, as the activity of mitochondrial uncoupling proteins can be induced by ROS. Both sod-2;3- and sod-1;4;5-deficient nematode strains were sensitive to arsenite (two way ANOVA, main effects of strain, arsenite, and their interaction (p<0.0001 for all)) (Figure 85). Finally, we tested for arsenite sensitivity in nematodes deficient in ucp-4 (the sole mitochondrial uncoupling protein homolog in nematodes). No sensitivity was observed compared to wild-type (N2) nematodes (two way ANOVA, main effect of arsenite (p<0.0001), but not strain (p=0.057), or their interaction (p=0.47)) (Figure 86). However, this may be explained by the fact that ucp-4 has been shown to play a role in succinate transport, and does not appear to function as a classical uncoupler in nematodes (193).

Because the arsenite-induced metabolic shift from oxidative phosphorylation to glycolysis has been shown to be dependent upon HIF-1A stabilization in several cell lines (154), we next tested whether hif-1a-deficient nematodes would be sensitive to arsenite. However, no sensitivity was observed in two different hif-1a-deficient strains (two way ANOVA, main effect of arsenite (p<0.0001), but not strain or their interaction (p>0.05 for both)) (Figure 87).
B.3.4 Mutations in ETC and glycolytic genes sensitize nematodes to arsenite exposure

Because both high and low concentrations of arsenite severely disrupted mitochondrial respiration and induced aerobic glycolysis, we hypothesized that mutations in glycolytic genes and various complexes of the ETC, which cause mitochondrial disease in humans (353), would sensitize nematodes to arsenite exposure. In support of our hypothesis, deficiencies in pyruvate kinase and lactate dehydrogenase sensitized nematodes to arsenite exposure in a larval growth assay (two way ANOVA, main effects of arsenite, strain and their interaction (p<0.0001 for all)) (Figure 88A), while deficiencies in ETC complexes I, II, and III, but not ATP synthase, also sensitize nematodes to arsenite exposure (two way ANOVA, main effects of arsenite, strain and their interaction (p<0.0001 for all)) (Figure 88B). Since many of these genetically deficient strains develop at reduced rates, all time of flight (ToF, a surrogate measure of nematode length) data was normalized to percent growth of control for statistical analysis (Figure 88; raw ToF data is shown in Figure 89).

B.3.5 Accumulation of arsenite in nematode mitochondria is comparable to arsenite accumulation in low-dose in vitro studies

Because the concentrations of arsenite used in this study are high compared to cell culture studies, we quantified the amount of arsenite accumulating in nematode mitochondria to determine if the concentrations of arsenite used in this study are comparable to low-dose (75ppb) in vitro studies that elicit similar effects. Concentrations
of arsenite in isolated mitochondria were 63.0 ± 6.5 and 330.1 ± 6.7 ng arsenic per mg mitochondrial protein following 48h exposure to 50 and 500µM arsenite, respectively (Figure 90), which, at least for 50µM, is comparable to concentrations of arsenite measured in mitochondria isolated from low-dose in vitro studies (301).

**B.4 Discussion**

Despite decades of research studying the toxic effects of arsenic, the mechanism by which arsenic causes cancer and other diseases remains poorly understood. Recently, arsenite was shown to induce aerobic glycolysis in vitro (154), implicating metabolic dysfunction in arsenic-induced disease. Here, using the model organism, *C. elegans*, we demonstrate that arsenite can induce glycolysis in vivo. Furthermore, we report for the first time arsenite-induced alterations in mitochondrial function, including reduced ATP-linked respiration and spare respiratory capacity, as well as increased proton leak. Finally, we report that deficiencies in the mitochondrial ETC, which cause disease in humans, sensitize nematodes to arsenite exposure, thus identifying a novel class of gene-environment interactions that warrant further investigation in the human populace.

Arsenite-induced aerobic glycolysis in vitro is dependent upon the stabilization of HIF-1A (154, 343, 354), a transcription factor that regulates expression of genes involved in glycolysis, and Krebs cycle activity (i.e. PDHK, a negative regulator of PDH) under hypoxic conditions (355). Under oxygen replete conditions HIF-1A is
constitutively targeted for proteasomal degradation via the action of prolyl hydroxylase domain (PHD) proteins and von Hippel Lindau proteins (356). Arsenite-induced ROS can deplete redox-sensitive ascorbate, a cofactor required for PHD function, leading to HIF-1A accumulation under oxygen replete conditions (354), an induction of aerobic glycolysis (154), and a loss of anchorage dependent cell growth (343). However, HIF-1A does not appear to be playing a role in the observed Warburg-like effect in nematodes, as HIF-1A-deficient strains were not sensitive to arsenite compared to wild type nematodes. Instead, the rapid disruption of pyruvate metabolism is likely due to reduced PDH activity. Because arsenite is a well-known inhibitor of PDH (253), direct enzyme inhibition could be responsible for reduced PDH activity; however, high intracellular ratios of ATP:ADP, acetyl-CoA:CoA, and NADH:NAD⁺ can also inhibit PDH activity, and cannot be ruled out.

Unexpectedly, arsenite did not alter basal respiration despite a dose-dependent reduction in ATP-linked respiration. This is surprising, as steady-state ATP levels were only reduced in nematodes exposed to 50µM arsenite. This suggests that an alternative pathway, such as glycolysis, is maintaining steady-state ATP levels. In agreement with this, 2-DG reduced steady-state ATP levels in glp-4 nematodes exposed to both 50 and 250µM arsenite; however, pyruvate and lactate only accumulated in glp-1 nematodes exposed to the highest concentration of arsenite. This could indicate a more robust induction of glycolysis, capable of maintaining ATP levels, in the highest exposure
group. Alternatively, limited lactate accumulation in nematodes could be explained by the presence of a pathway homologous to the mammalian Cori cycle, which converts lactate to glucose in the liver under periods of high energy demand. Although this pathway has not yet been confirmed, the *C. elegans* genome contains a monocarboxylate transporter homolog (K05B2.5), which functions as a lactate transporter in the mammalian Cori cycle (357). Interestingly, under anoxic conditions, nematodes do not appear to accumulate lactate in their tissues. Instead, excess lactate is excreted or converted to ethanol and then excreted (358, 359), which could also explain why lactate did not accumulate in nematodes exposed to lower concentrations of arsenite.

Arsenite exposure induced a dose-dependent increase in proton leak, which is catalyzed biologically in part via the action of uncoupling proteins, whose activity can be induced via superoxide, fatty acid anions, and by-products of lipid peroxidation. Thus, inducible proton leak can function to limit further mitochondrial ROS production through a negative feedback loop (18). Indeed, reduced mitochondrial membrane potential following arsenite exposure is supportive of mitochondrial uncoupling. However, consistent with previous reports that *ucp-4*, the single known *C. elegans* uncoupling protein homolog, does not function as a classical uncoupler (193), *ucp-4*-deficient nematodes were not sensitive to arsenite exposure, compared to wild type nematodes. The fact that both mitochondrial (*sod-2;3*) and non-mitochondrial (*sod-1;4;5*) superoxide dismutase-deficient nematodes were sensitive to arsenite provides evidence
of arsenite-induced superoxide production, which could then induce proton leak through other mitochondrial carriers, such as the adenine nucleotide translocator or dicarboxylate carrier, which facilitates fatty acid-mediated proton transport (360). In support of this, arsenite has previously been shown to induce ANT channel activity in vitro (361).

Spare respiratory capacity was severely reduced by both 50 and 500µM arsenite. This could be explained by the fact that mitochondrial respiration is already uncoupled by arsenite, thus limiting further uncoupling by FCCP. Furthermore, arsenite exposed nematodes were less sensitive to ATP depletion in the presence of the mitochondrial uncoupler, FCCP, which further supports arsenite-induced mitochondrial uncoupling, and an alternative, non-oxidative, means of ATP production. Alternatively, arsenite may reduce SRC by directly inhibiting complexes of the ETC or by reducing Krebs cycle activity, thus limiting ETC substrate (NADH, FADH₂). However, only methylated trivalent arsenicals (MMA and DMA) have been shown to directly inhibit the ETC (301), and as the nematode genome lacks an AS3MT homolog (322), direct inhibition seems unlikely. Finally, although the accumulation of pyruvate suggests reduced flow of pyruvate into the Krebs cycle, and thus reduced Krebs cycle activity, no Krebs cycle intermediates were significantly altered by arsenite exposure, ostensibly eliminating substrate limitation as causative of reduced SRC.
In addition to the oxidation of glucose, the oxidation of fatty acids and amino acids can also fuel mitochondrial respiration. However, only minor changes in acylcarnitine levels, a biomarker of acyl-CoA species (352), were observed following arsenite exposure, suggesting arsenite has minimal effects on fatty acid oxidation. Interestingly, both 50 and 500µM arsenite significantly increased the levels of a majority of amino acids (10/15), a change consistent with increased autophagy and/or proteasomal degradation. This is further supported by the fact that \textit{bec-1-} and \textit{atg-18-} deficient nematodes are sensitive to arsenite exposure. Arsenite induced autophagy is not surprising, as arsenite has high affinity for protein thiols (251), and thiol binding has been shown to cause protein misfolding (362) and subsequent proteasomal (253) and lysosomal degradation (363). Furthermore, the accumulation of ubiquitin-conjugated proteins (364), and the induction of autophagy (246, 247) following arsenite exposure have previously been reported \textit{in vitro}. Alternatively, alanine, ornithine, and citrulline appear to be the amino acids most effected by arsenite exposure. This further suggests altered pyruvate metabolism (i.e. increased transamination of pyruvate to alanine), and/or disruption of the urea cycle, or the nematode equivalent, as nematodes appear to lack functional urea cycle enzymes (351).

Genetic variation is associated with altered arsenic-induced disease and cancer risk (365). However, little is currently known about how individuals suffering from mitochondrial disease will respond to arsenite exposure. We were concerned that
individuals suffering from mitochondrial disease could represent a large subpopulation displaying increased sensitivity to arsenite, as collectively, an estimated 1 in 4,000 individuals suffer from mitochondrial disease (129). Indeed, we observed increased arsenite sensitivity in nematodes deficient in ETC complex I (nuo-6), II (mev-1), and III (isp-1, isp-1;ctb-1), but not V (atp-2). nuo-6, isp-1, isp-1;ctb-1, and atp-2 nematodes are long-lived and ROS-resistant, whereas mev-1 nematodes are short-lived and ROS-sensitive (185, 238, 366). Interestingly, many ETC mutants upregulate the activity other ETC complexes to survive, for example complex I mutants have been shown to upregulate complex II (367). Thus, it is likely that reduced metabolic plasticity in the context of arsenite-induced inhibition of complexes I, II and V (Figure 75), and not hypersensitivity to ROS, underlies the observed genetic sensitization to arsenite toxicity. Interestingly, previous studies have shown mild uncoupling in yeast and Chlamydomonas carrying ATP-synthase deficiencies (368, 369), which may help to limit arsenite toxicity in atp-2-deficient nematodes.

Presently, it is unclear why PE255 glp-4 nematodes are more sensitive to arsenite than glp-1 nematodes. Although mutations in both genes result in similar germline phenotypes, they participate in different molecular processes: glp-1 encodes a notch receptor ligand (370), whereas glp-4 encodes a valyl aminoacyl tRNA synthetase (371). This raises the possibility that glp-4 plays a role in arsenic-related toxicity. However, we have previously reported a similar differential sensitivity between the two strains to 5-
fluoro-2′-deoxyuridine (DNA synthesis inhibitor) (326), and have observed the same for rotenone and paraquat (unpublished observations). Furthermore, arsenic induces similar alterations in mitochondrial respiration between the two strains (i.e. increased proton leak, and reduced ATP-linked OCR and spare capacity). These results demonstrate that increased sensitivity in glp-4 nematodes is not limited to arsenite, and that arsenite induces similar trends in mitochondrial dysfunction between the two strains.

Although the concentrations of arsenite used in this study are high (3.75ppm and 37.5ppm), drinking water arsenic concentrations in the 1-5ppm range have been measured in Argentina, Bangladesh, Chile, Thailand, Vietnam, and West Bengal (334). More importantly with regard to the relevance of our study, nematodes display some of the lowest sensitivity to arsenic yet observed, with a previously reported 24h LC₅₀ value of 1.3mM arsenite (256, 282), although here we report a slightly higher 24h LC₅₀ value of ~5mM. Reduced sensitivity to arsenite is further demonstrated by the fact that only chronic, lifelong exposure to ≥100µM (~7.5ppm) arsenite reduce nematode lifespan (~10-15%) (372), while chronic exposure to much lower concentrations (10-150ppb) of arsenite are associated with increased mortality rates in humans (335). Reduced sensitivity is likely, in part, due to the nematode’s cuticle, a collagenous barrier that has been shown to limit the absorption of a variety of toxicants, including: bisphenol A (297), oligomycin (228), and drugs such as nicotine and ivermectin (163). The nematode cuticle is also
impermeable to metal ions such as manganese (281), thus, is likely also impermeable to arsenite. Importantly, the internal dose of arsenite inducing the Warburg effect in vitro and in vivo is similar, as the amount of arsenite in nematode mitochondria (48h exposure to 50µM arsenite) and rat liver cell (RLC16) mitochondria (24h exposure to 1µM arsenite) (301) is similar (63 vs 5ng arsenite/mg protein, respectively). Another point to consider is that it takes several weeks of exposure to 1µM arsenite to induce the Warburg effect in vitro (154), whereas a Warburg-like effect was observed in nematodes after 48h of arsenite exposure.

**B.5 Conclusions**

Using a combination of novel and established methods including toxicological, biochemical, and genetic techniques, we report that arsenic results in metabolic shifts in vivo that are consistent with reported roles of arsenic in carcinogenesis (343), metabolic syndrome (222, 223), and other metabolism-related pathologies (221, 340, 341). In particular, we report an arsenite-induced Warburg-like effect which has previously only been demonstrated in vitro, as well as previously unidentified alterations in mitochondrial respiration (reduced SRC and ATP-linked respiration, and increased proton leak). Finally, we demonstrate that deficiencies in ETC complexes I, II, and III sensitize nematodes to arsenite exposure, thus identifying a novel class of gene-environment interactions that warrant further investigation for their human relevance.
B.6 Acknowledgements

We thank Elena Turner and Jonathan Hibshman for their advice and assistance in the extraction of small metabolites for metabolomics analysis.
Table 8: Preparation of Inhibitors for Metabolic Inhibition Assay.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Target</th>
<th>Incubation Length (hr)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Initial Concentration (8X)</th>
<th>Final Concentration (%DMSO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% DMSO</td>
<td>Control</td>
<td>1</td>
<td>16%</td>
<td>2%</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>ETC Complex IV</td>
<td>1</td>
<td>80mM (100% EPA H&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>10mM (100% EPA H&lt;sub&gt;2&lt;/sub&gt;O)</td>
</tr>
<tr>
<td>FCCP</td>
<td>Mitochondrial Uncoupler</td>
<td>1</td>
<td>200µM (16%)</td>
<td>25µM (2%)</td>
</tr>
<tr>
<td>1% DMSO</td>
<td>Control</td>
<td>3</td>
<td>8%</td>
<td>1%</td>
</tr>
<tr>
<td>Rotenone</td>
<td>ETC Complex I</td>
<td>3</td>
<td>80µM (8%)</td>
<td>10µM (1%)</td>
</tr>
<tr>
<td>TTFA</td>
<td>ETC Complex II</td>
<td>3</td>
<td>8mM (8%)</td>
<td>1mM (1%)</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>ETC Complex III</td>
<td>3</td>
<td>80µM (8%)</td>
<td>10µM (1%)</td>
</tr>
<tr>
<td>DCCD</td>
<td>ATP Synthase</td>
<td>3</td>
<td>160µM (8%)</td>
<td>20µM (1%)</td>
</tr>
<tr>
<td>DCA</td>
<td>Pyruvate Dehydrogenase Kinase</td>
<td>4.5</td>
<td>8mM (100% EPA H&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>1mM (100% EPA H&lt;sub&gt;2&lt;/sub&gt;O)</td>
</tr>
<tr>
<td>2-DG</td>
<td>Glycolysis</td>
<td>4.5</td>
<td>400mM (100% EPA H&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>50mM (100% EPA H&lt;sub&gt;2&lt;/sub&gt;O)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Inhibitor incubation periods were chosen based on Seahorse XF<sup>e</sup> Bioanalyzer experiments, while a 4.5h incubation was chosen for 2-DG as this incubation period resulted in the greatest depletion of ATP in the context of arsenite exposure (data not shown).
Panels B-H show the percent ATP remaining in arsenite treated nematodes after exposure to the vehicle control or various metabolic inhibitors. Higher percent ATP remaining indicates a reduced role in steady state ATP maintenance, and thus reduced activity, for the complex being inhibited. (A) A 48h exposure to arsenite reduced steady-state ATP levels in a dose-dependent manner (one way ANOVA, p<0.0001). Exposure to (B) 1% DMSO (3h exposure) caused a slight reduction (16%) in ATP in nematodes exposed to 250µM arsenite (one way ANOVA, p=0.0085); however, no sensitivity to (C) 2% DMSO (1h exposure) was observed (one way ANOVA, p=0.25). A trend in reduced (D) complex I activity was observed in nematodes exposed to arsenite (one way ANOVA, p=0.0031).
ANOVA, p=0.069), whereas arsenite reduced the activity of (E) complex II (one way ANOVA, p<0.0001), (F) ATP synthase (one way ANOVA, p<0.0001), and reduced sensitivity to (G) mitochondrial uncoupling (one way ANOVA, p=0.038), while increasing (H) glycolysis (one way ANOVA, p=0.017). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=4-6. Bars ± SEM.
Inhibition of ETC complexes (A) III (one way ANOVA, p=0.75), (B) IV (one way ANOVA, p=0.99), or (C) pyruvate dehydrogenase kinase (one way ANOVA, p=0.20) had no effect on ATP levels in arsenite treated nematodes compared to controls. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD test) to control. N=3. Bars ± SEM.
Figure 77: Arsenite disrupts pyruvate metabolism.

Exposure to 500µM arsenite resulted in the accumulation of (A) pyruvate after 12 (one way ANOVA, $p=0.0088$), 24 (one way ANOVA, $p=0.0011$), and 48h (one way ANOVA, $p=0.0002$) of exposure; whereas (B) lactate levels were only affected after 48h of arsenite exposure (one way ANOVA, $p=0.048$). Alternatively, exposure to both 50 and 500µM arsenite reduced pyruvate dehydrogenase (PDH) activity after 48h of exposure (one way ANOVA, $p=0.0016$). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey’s HSD) to control within each time point. N=4-6. Bars ± SEM.
Figure 78: Arsenite alters amino acid levels.

Amino acid levels were elevated following 48h of exposure to arsenite. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=5. Bars ± SEM.
Figure 79: Deficiencies in autophagy sensitize nematodes to arsenite.

(A) *bec*-1-deficient nematodes are sensitive to arsenite exposure compared to wild type (N2) nematodes (two way ANOVA, main effects of strain (p<0.0001), arsenite (p<0.0001), and their interaction (p=0.013). (B) 800µM Arsenite reduced N2 nematode growth (one way ANOVA, p=0.0008), whereas both 600 and 800µM arsenite reduced *bec*-1 growth (one way ANOVA, p=0.0002). Data is represented as (A) percent growth of control, and (B) ToF, a surrogate for nematode length. Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to (A) N2 at each arsenite concentration and (B) the untreated control within each strain. N=165-406. Bars ± SEM.
Figure 80: Deficiencies in autophagy sensitize nematodes to arsenite.

L4 bec-1- and atg-18-deficient nematodes are exhibit greater sensitivity to arsenite compared to wild type (N2) nematodes in a 24h lethality assay (two way ANOVA, main effects of strain, arsenite, and their interaction (p<0.0001 for all). Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to N2 at each arsenite concentration. N=6. Bars ± SEM.
Figure 81: Arsenite does not alter genome copy number.

Arsenite exposure does not alter (A) nuclear (one-way ANOVA, p=0.99) or (B) mitochondrial (one-way ANOVA, p=0.25) genome copy number, or their (C) ratio (one-way ANOVA, p=0.87). N=9. Bars ± SEM.
Figure 82: Arsenite disrupts mitochondrial respiration.

(A) Metabolic profile obtained via Seahorse XF24 analysis following a 48h exposure to arsenite. Arsenite reduced (B) ATP levels (one-way ANOVA, p=0.042, N=9-12), (C) ATP-linked respiration (one-way ANOVA, p<0.0001, N=22), (D) spare respiratory capacity (one-way ANOVA, p<0.0001, N=22), and increased (E) proton leak (one-way ANOVA, p<0.0001, N=22) in glp-1 nematodes. Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to control. Bars ± SEM.
Figure 83: Arsenite disrupts the fundamental parameters of mitochondrial respiration in PE255 glp-4-deficient nematodes.

(A) Metabolic profile obtained via Seahorse XF-24 analysis following a 48h exposure to arsenite. Arsenite reduced (B) ATP-linked respiration (one-way ANOVA, p=0.0002, N=11), (C) spare respiratory capacity (one-way ANOVA, p=0.025, N=11), and increased (D) proton leak (one-way ANOVA, p=0.018, N=11). Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to control. Bars ± SEM.
Figure 84: Arsenite reduces mitochondrial membrane potential.

The ratio of red to green fluorescence intensity was measured in the posterior pharyngeal bulb of (A) control, (B) 50μM FCCP (positive control), (C) 50μM arsenite, and (D) 500μM arsenite exposed nematodes. (E) Arsenite reduced mitochondrial membrane potential in a concentration dependent manner (one-way ANOVA, p=0.0023). Panels A-D show representative images of JC-1 staining. Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to control. N=8. Bars ± SEM.
Figure 85: Deficiencies in superoxide dismutase sensitize nematodes to arsenite.

Mitochondrial iron/manganese double mutants (sod-2;3), and cytoplasmic and extracellular copper/zinc superoxide dismutase triple mutants (sod-1;4;5) are sensitive to arsenite compared to wild type (N2) nematodes in a 24h lethality assay (two way ANOVA, main effects of strain, arsenite, and their interaction (p<0.0001 for all). Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to N2 at each arsenite concentration. N=6. Bars ± SEM.
Figure 86: *ucp*-4-deficient nematodes are not sensitive to arsenite.

(A) *ucp*-4-deficient nematodes display no sensitivity to arsenite, compared to wild-type (N2) nematodes, in a 48h larval growth assay (two way ANOVA, main effect of arsenite (p<0.0001), but not strain (p=0.057) or their interaction (p=0.47)). (B) 800µM arsenite reduced both N2 (one way ANOVA, p=0.0008) and *ucp*-4-deficient nematodes (one way ANOVA, p=0.0003) growth. Data is represented as (A) percent growth of control, and (B) ToF, a surrogate for nematode length. Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to the untreated control within each strain. N=178-406. Bars ± SEM.
Figure 87: Deficiencies in hypoxia inducible factor 1A do not sensitize nematodes to arsenite.

hif-1α-deficient strains (ia4 (deletion) and ia7 (point mutation)) displayed no sensitivity to arsenite compared to wild type (N2) nematodes (Two way ANOVA, main effect of arsenite (p<0.0001), but not strain or their interaction (p>0.05 for both). N=6. Bars ± SEM.
Nematodes deficient in (A) pyruvate kinase and lactate dehydrogenase (two way ANOVA, main effects of strain, arsenite and their interaction (p<0.0001 for all)), as well as (B) ETC complexes I (nuo-6), II (mev-1), and III (isp-1; isp-1:ctb-1), but not ATP synthase (atp-2), are sensitive to arsenite exposure (two way ANOVA, main effects of strain, arsenite and their interaction (p<0.0001 for all)) in a 48h larval growth assay. As nematode strains develop at varying rates, all ToF (surrogate for nematode length) data is normalized to percent growth of control. Raw ToF data is shown in Figure 89. Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to N2 within each arsenite concentration, and are listed in Supplemental File 1. N=166-406. Bars ± SEM.
Figure 89: Deficiencies in glycolysis and ETC genes sensitize nematodes to arsenite.

(A) Arsenite reduced N2 (one way ANOVA, p=0.0008), idh-1- (one way ANOVA, p<0.0001), and pyk-1- (one way ANOVA, p<0.0001) deficient nematode growth in a 48h larval growth assay. (B) Arsenite reduced N2 (one way ANOVA, p=0.0008), nuo-6- (one way ANOVA, p<0.00001), mev-1- (one way ANOVA, p<0.0001), isp-1- (one way ANOVA, p<0.0001), isp-1:ctb-1- (one way ANOVA, p<0.0001), but not atp-2- (one way ANOVA, p=0.082) deficient nematode growth in a 48h larval growth assay. Time of flight (ToF) is a surrogate measure of nematode length. Asterisk denotes statistical significance (p<0.05) for post-hoc (Tukey’s HSD) comparison to the untreated control within each strain. N=166-406. Bars ± SEM.
Figure 90: Arsenic accumulates in mitochondria.

Arsenite accumulates in mitochondria isolated from glp-1 nematodes exposed to 0, 50, or 500µM arsenite for 48h (one way ANOVA, p<0.0001). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars ± SEM.

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C.1 Introduction

Mitochondria, best known for producing ATP via oxidative phosphorylation (OXPHOS), also house all or part of many other important metabolic pathways such as the Krebs cycle (128) and fatty acid β-oxidation (373), making them the nexus of intermediary metabolism. Therefore it is not surprising that mitochondrial dysfunction is causal and/or implicated in myriad human diseases, including cancer (374-376), neurodegeneration (377, 378), and metabolic disorders (379). In fact, as many as 1 in 5,000 individuals are estimated to be afflicted by mitochondrial disease (129). Furthermore, mitochondria integrate environmental and intercellular signals to meet cellular energy demands (35). Many pharmacological (132) and environmental toxicants (66) are known mitochondrial poisons that can interfere with this signaling. Understanding how genetics, toxicant exposure and gene-environment interactions affect mitochondrial function in vivo will be crucial in preventing, diagnosing and treating mitochondrial disease in the future.
The model organism *Caenorhabditis elegans*, a free-living, multicellular nematode, offers many advantages over other model organisms for studying mitochondrial function *in vivo*. Ease and low cost of maintenance, a short life cycle (~2 weeks), and a high reproductive rate (~300 offspring per wild-type nematode) make *C. elegans* a powerful model for medium-throughput experiments (137, 148). Furthermore, a fully sequenced and annotated genome (140), conserved biochemical pathways (139) and mitochondrial biology (112), and availability of genetic mutants (141) and simple RNAi knockdown technology (142, 143) make *C. elegans* a good model for studying mitochondrial function *in vivo*. Current tools for assessing mitochondrial health in nematodes include *in vivo* analysis of mitochondrial morphology (380) and ATP levels (145) using transgenic reporter strains, oxygen consumption via low throughput Clarke-type electrode oxygen meters (146), and time consuming biochemical analysis of extracts (144). Here, using the Seahorse XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Massachusetts, USA) we describe how to measure the fundamental parameters of the electron transport chain (ETC): basal oxygen consumption rate (OCR), ATP-linked respiration, maximal OCR, spare respiratory capacity, and proton leak.

**C.2 Basic protocol**

**C.2.1 in vivo quantification of the fundamental parameters of the mitochondrial electron transport chain in larval stage four nematodes**

Using the pharmacological inhibitors dicyclohexylcarbodiimide (DCCD, ATP synthase inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP,
mitochondrial uncoupler) and sodium azide (cytochrome c oxidase inhibitor), we describe how to measure the fundamental parameters of the mitochondrial ETC, including basal OCR, ATP-linked respiration (basal OCR minus DCCD inhibited OCR), maximal OCR (FCCP-induced OCR), spare respiratory capacity (FCCP-induced OCR minus basal OCR), and proton leak (DCCD inhibited OCR minus sodium azide inhibited OCR), in vivo, in larval stage four (L4) C. elegans. Although we outline this assay for use with L4 nematodes, as the L3/L4 transition is accompanied by a metabolic switch from glycolysis to oxidative phosphorylation (112, 151-153), this protocol can easily be adapted for analysis of mitochondrial function in older and/or younger nematodes, mutant strains, or for mitochondrial toxicity testing, as outlined throughout the protocol.

C.2.2 Materials

- XF Flux pack. Contents include:
  1. 18 XF-24 extracellular flux assay kits (each kit contains a sensor cartridge, lid, hydrobooster, and 24-well utility plate)
  2. 500 mL XF Calibrant
  3. 20 XF cell culture microplates

- OP50 K-agar plates with synchronous populations of L4 nematodes (see support protocol 1)

- 2.0mM DCCD (dissolved in DMSO, store at -20°C)
• 1.25mM FCCP (dissolved in DMSO, store at -20°C)

• 80mM sodium azide (dissolved in unbuffered EPA water (see recipe), store at 4°C)

• 0.1% Triton X-100 (diluted in ddH₂O, store at room temperature)

• 10% Sodium dodecyl sulfate (SDS) solution (dissolved in ddH₂O, store at room temperature for up to 6 months)

• K-medium (see recipe)

• Unbuffered EPA water

• Thermo Scientific™ Pierce™ BCA™ Protein Assay BCA Assay

• 15mL Centrifuge tubes

• Microscope Slides

**C.2.3 Equipment**

• Seahorse XFe24 Extracellular Flux Analyzer & computer (provided with flux analyzer with XF®24 Wave software installed)

• Orbital shaker

• 20°C incubator

• Dissecting light microscope

• Ultrasonicator (Ultrasonic homogenizer, Model 3000, Biologics, Inc., Virginia, USA)
• Microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany, with filters capable of measuring absorbance in the range of 540-590nm)

C.2.4 Hydrating the Seahorse XFe24 sensor cartridge

1. Pipette 1.0mL of XF Calibrant solution into each well of a 24-well utility plate, then place the hydro-booster (pink spacer) onto the utility plate, followed by the sensor cartridge and finally the lid. Place the sensor cartridge out of direct sunlight to hydrate for a minimum of 4 hours.

   IMPORTANT. The sensor cartridge must be left hydrating for a minimum of 4 hours prior to use. We typically allow the sensor cartridge to hydrate overnight (12-18 hours) in a 25°C incubator, but have successfully used cartridges that have been left to hydrate for up to 4 days at 25°C. A diagram of how to hydrate a sensor cartridge is provided by the manufacturer with each XF Flux pack.

C.2.5 Nematode culturing

2. Using a Pasteur pipette, transfer synchronized populations of L1 nematodes, obtained by hypochlorite bleaching (Supporting Protocol 1), onto OP50 seeded k-agar plates at 20°C. Incubate the nematodes at 20°C until a synchronous population of L4 nematodes is obtained.

   In order to obtain a synchronous population of L4 nematodes, the OP50 food source should never be allowed to become depleted; developing nematode
populations should be transferred to fresh OP50 k-agar plates as necessary. This is of particular importance in the context of measurement of metabolic function, because nematodes respond to food scarcity by altering their metabolic programming.

We find that synchronous L1 wild-type N2 nematodes reach L4 after being incubated with OP50 at 20°C for 48 hours. However, if you are working with growth delayed strains, a longer incubation period may be required to obtain a synchronous population of L4s. For example, complex III mutant isp-1 (qm150) requires ~96h to reach L4 and outer/inner mitochondrial fusion-deficient fzo-1 (tm1133) and eat-3(ad426) nematodes require incubation with OP50 at 20°C for 72h.

C.2.6 Seahorse XFe24 Analyzer WAVE software set-up

3. Turn on the Seahorse XF24 and start the Wave software.

4. In the WAVE software click on the “New” tab, then highlight the “Blank” option and click “Design” to start designing a new assay.

5. Click the “Assay Conditions” tab, followed by the “Cell Type” tab, and then click “Add Cell Type Condition”. Add the appropriate information to the Name (strain, larval stage, treatment etc.), Cell Type (C. elegans and/or strain name) and Seeding Density (number of nematodes added per well) fields.
If more than one strain or treatment is being tested in the assay you can add additional cell types by clicking on the “Cell Type” heading and then clicking “Add”. This allows you to name each cell type as necessary.

We typically run each Seahorse plate with 4 strains or treatments, such that each strain or treatment is run in 5 to 6 wells of an islet plate. Also note that two wells must be designated as blanks (we typically use wells A1 and D6, although any wells can be used for this purpose), leaving 22 wells available for samples.

6. Click on the “Injections” tab, followed by “Add Injection Strategy”, then highlight port A and click “Add”. Add the appropriate information to the Compound (i.e. FCCP, DCCD, or sodium azide), Concentration (final drug concentrations are 25µM, 20µM, and 10mM for FCCP, DCCD, and sodium azide, respectively), Solvent (i.e. DMSO) and Percent Solvent (final percent DMSO is 2, 1, or 0% for FCCP, DCCD, and sodium azide, respectively) fields.

Drugs cannot be injected in tandem from the sensor cartridge. We have found that the magnitude of sodium azide’s effect is significantly reduced if injected post-FCCP (228). It may be possible to inject another complete respiratory inhibitor (i.e. rotenone and antimycin A or cyanide) post-FCCP injection, however this has not been tested. Likewise, it may also be possible to inject sodium azide after DCCD, increasing sample throughput.
We have also found that sensor cartridges cannot be reused after drug injection. For example, basal OCR is elevated in nematodes when a sensor cartridge previously used for FCCP injection is used. Thus we test each drug in its own new sensor cartridge.

7. Click “Generate Groups” and then click on the “Plate Map” tab. Assign the groups to the plate map by clicking on the first group (i.e. control or wild-type strain) and then click on the wells of the 24-well plate map where you will load the group into the 24-well islet plate. Assign all of your groups an equal number of wells on the plate map, leaving 2 wells as blanks (again, we typically use A1 and D6).

Groups should be assigned randomly to an equal number of wells across the plate to avoid potentially confounding plate effects.

8. Select the “Instrument Protocol” tab to set up the OCR measurements protocol. Click on the “Edit Measurement Details” dropdown tab under the “Basal Measurement Cycles” heading and change the number of cycles to 8, the mix cycle to 1 (to oxygenate the micro-chamber), the wait cycle to 3 (to allow nematodes to settle) and the measure cycle to 3 minutes.

The mix cycle is very important. Oxygen levels will fall during the 3 minute measurement cycle, but should not fall far below 100mmHg. If oxygen levels do fall below 100mmHg, then they should recover after the mix cycle (~160mmHg). If oxygen levels do not recover, it is a good indication that too
many nematodes have been loaded per well (Kevin Bittman, Ph.D., Field Applications Scientist, Seahorse Biosciences, Inc., personal communication).

9. Click the “Injection” button, select the port that you will be injecting your drug from (typically port A), and name your injection strategy (i.e. 25µM FCCP, 20µM DCCD or 10mM sodium azide). Click on the “Edit Measurement Details” dropdown tab under the injection strategy heading and change the number of cycles.

*The number of cycles will vary depending on which inhibitor you are injecting.*

*We use 8, 14, or 4 measurement cycles to measure nematode response to FCCP, DCCD, or sodium azide respectively. The Mix (1 minute), Wait (3 minutes), and Measure (3 minutes) cycle times mentioned in step 8 do not change.*

10. Click on the “Review and Run” tab. Name and save the assay.

### C.2.7 Preparation of ETC inhibitors for injection

11. Prepare stocks of 80mM sodium azide in EPA water, 1.25mM FCCP in DMSO, and 2mM DCCD in DMSO.

*Store single use aliquots of 350µl 1.25mM FCCP and 200µl 2mM DCCD at -20°C to limit freeze thawing your stocks of the inhibitors. 80mM sodium azide should be stored at 4°C.*

12. Dilute the ETC inhibitor stocks to the appropriate port concentration as described in Table 9.
13. Pipette 75µl (8X concentrated) of the desired ETC inhibitor into the appropriate injection ports of the sensor cartridge.

75µl of the appropriate ETC inhibitor are loaded into the sensor cartridge port at an 8X concentration. Thus, for example, when 75µl of 200 µM FCCP in 16% DMSO are injected into a well containing nematodes in 525µl EPA water, it is diluted to a final working concentration of 25µM in 2% DMSO (i.e. 1:8 dilution).

14. Under the “Review and Run” tab click “Start Run” and the Seahorse Analyzer’s tray will slide out. Remove the lid and hydro-booster and place the sensor cartridge and utility plate on the Seahorse Analyzer’s tray. Click “ready” to start calibrating the sensor cartridge.

The calibration process takes approximately 20 minutes. During this time, you will add your synchronous L4 nematodes to a Seahorse Analyzer islet plate (step 15).

C.2.8 Preparation of nematodes for Seahorse XF\textsuperscript{e}24 Analyzer assay

15. Using sterile k-medium wash your synchronized population of L4 nematodes (from step 2) off of an OP50 seeded k-agar plate into a 15mL centrifuge tube. Centrifuge 30 seconds at 2200 RCF or let nematodes settle by gravity (this is most effective with larger nematodes). Aspirate the supernatant. Resuspend the nematodes in 10mL k-medium, and repeat the pelleting and rinsing process.
This step removes live OP50 from the medium that could otherwise confound OCR measurements. This issue could also be overcome by feeding nematodes either heat- or UVC-inactivated bacteria.

16. After the second k-medium rinse, resuspend the nematodes in 10mL k-medium and place the centrifuge tube on an orbital shaker at 20°C for 20 minutes.

This provides the nematodes time to clear live OP50 from their guts, which could confound OCR measurements.

17. After the 20 minute incubation, allow the nematodes to settle by gravity or centrifuge for 30s at 2200 RCF. Aspirate the supernatant. Resuspend the nematodes in unbuffered EPA water to a concentration of 1 ± 0.2 nematodes per microliter (step 18).

18. To estimate the number of nematodes per microliter, invert the centrifuge tube 2-3 times to suspend the nematodes, then pipette 20µl of nematodes onto a glass slide and count the number of nematodes in 3-4 20µl drops using a dissecting light microscope. Calculate the average number of nematodes per microliter and the volume required to obtain 75 and 1000 nematodes (i.e. if you have on average 0.9 nematode/µl, then 83µl and 1111µl will contain ~75 and ~1000 nematodes, respectively).
We trim the tip of each pipette tip with scissors and use a new tip rinsed in 0.1% triton X-100 (i.e. pipette and exspell the triton prior to pipetting nematodes) for each 20µl drop. The triton X-100 helps prevent nematodes from sticking to the pipette tips, while trimming the tips increases the circumference, allowing large worms to be pipetted without injury. This helps ensure an accurate nematode/µl estimation.

19. Following the plate map (step 7), pipette the volume required for 75 L4 nematodes into each well of a 24-well Seahorse XF24 islet plate and bring the volume of each well to 525µl with unbuffered EPA water.

When adding nematodes to the islet plate, be sure to use a new, trimmed pipette tip rinsed in 0.1% triton X-100 for each well of the 24-well islet plate. 

Cap and invert the 15mL centrifuge tube 2-3 times inbetween wells to ensure nematodes are fully resuspended.

20. By this time the sensor cartridge will be done calibrating (step 14). In the WAVE software click “Ready” and the Seahorse Analyzer’s tray will slide out with the utility plate. Replace the utility plate with the nematode-containing islet plate (without lid) and click “Ready” to start the assay.

In this step only the utility plate slides out with the tray, the sensor cartridge does not. With current Seahorse Analyzer settings the runtime for the
FCCP, sodium azide, and DCCD plates are roughly 150, 120, and 210 minutes, respectively.

21. Using trimmed pipette tips rinsed in 0.1% triton X-100, pipet the volume required for ~1000 L4 nematodes into a microcentrifuge tube and freeze at -80°C until total protein analysis in step 24.

22. Upon completion of the assay, click “Okay” and the analyzer’s tray will slide out. Remove the sensor cartridge and islet plate from the tray, click “Okay”, and the tray will slide closed. You will next be asked if you want to open the results from the current assay, click “Yes”. As the islet plate and sensor cartridge came into contact with toxic substances they should be disposed of according to your institution’s toxic waste disposal guidelines. The utility plate can be thrown directly into the trash.

23. Click on the small box in the upper left hand side of the 24-well plate map to display all OCR data in the graphed results section. Make sure background correction is turned on and then click on the “Display” dropdown and select “well”. Right click on the graph and select “Export Graph Data” to save the excel file. This will generate an excel file containing all of the OCR measurements taken for each well of the plate. Wells will be labeled A1-D6 in the file, so be sure to re-label the data with the appropriate strain/treatment conditions for each well, as assigned in the plate map (step 7).
C.2.9 Total Protein Extraction

24. Remove the aliquot of 1000 nematode from the -80°C freezer (step 21). Let the samples thaw at room temperature and then spin at 2200 RCF for 1 minute. Remove the supernatant, resuspend the nematodes in 250ul 10% SDS, and chill on ice for 2-3 minutes.

   10% SDS freezes rapidly when on ice. However, sonication (step 25) heats the samples, so it is best to start the sonication process with pre-chilled samples. If samples do freeze let them sit at room temperature until they re-thaw.

25. Ultrasonicate each chilled sample (on the lowest possible setting) for 30 seconds. Chill on ice for 2-3 minutes and then ultrasonicate for an additional 30 seconds.

   After the second 30s sonication period, pipette 10ul of the homogenate onto a slide under a microscope to ensure complete homogenization. No large debris should be present. If necessary sonicate for an additional 30s.

   IMPORTANT! Wipe the ultrasonicator micro-tip down with 70% ethanol in between every samples to prevent protein carryover between samples.

26. When complete homogenization is achieved, measure protein content using the BCA assay (Thermo Fisher Scientific, Rockford, IL), following manufacturer’s instructions.
Be sure to use 10% SDS when preparing your standard curve (see manufacturer’s instructions). This assay gives protein values in units of µg/mL, convert to mg/mL for normalization purposes.

The BCA assay requires access to a microplate reader with filters capable of measuring absorbance in the range of 540-590nm. We use a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany); however, any microplate model, with the proper filters, will work.

C.2.10 Data Analysis

27. Open the excel file from step 23 and average the readings from each well as follows:

1) **Basal OCR**: Readings 1-8 for each well. Values tend to be variable over the initial 4 measurements, so average OCR measurements 5-8 to get an average basal OCR per well (units of pmol O₂/minute).

2) **DCCD response**: OCR readings 9-22 for each well are for the 14 DCCD injection readings. Nematodes do not respond to DCCD instantly. It typically takes 8 measurements before OCR levels fall and plateau; thus, we average the final 6 OCR measurements (measurements 17-22) to get an average DCCD response per well.

3) **FCCP response**: OCR readings 9-16 for each well are for the 8 FCCP injection readings. Nematodes do not respond to FCCP instantly; however, OCR tends to increase and plateau by the fourth measurement. Thus we average the
final 5 OCR measurements (measurements 12-16) post-FCCP injection to get an average FCCP-response per well.

4) Sodium azide response: OCR readings 9-12 for each well are for the 4 azide injection readings. Nematodes respond to sodium azide essentially instantaneously, so we average all 4 OCR readings post-azide injection to calculate the average azide response per well.

28. Divide the average basal and the average drug-response OCR measurements by the number of nematodes added per well (i.e. 75) to convert OCR measurements to units of pmol O\textsubscript{2}/min/nematode. Next, divide your OCR measurements for each well by the corresponding total protein values calculated in step 26 to get units of nmol O\textsubscript{2}/min/mg protein.

Using this method we count each well measured as an n of one. We recommend repeating each experiment 2-3 separate times (i.e., with entirely separate cultures of nematodes).

NOTE: Depending upon the researcher's interests, nematode OCR data can be normalized a variety of ways, other than to total protein, including: worm number, worm volume, or mitochondrial mass or volume.

29. Calculate spare capacity, ATP-linked respiration, and proton leak as follows:

1) **Spare respiratory capacity** (Maximal OCR - Basal OCR) is the ability of an organism to respond to increasing energy demands. To calculate, subtract
each well’s average basal OCR from its corresponding average FCCP response (maximal OCR). Values from across experiments can be averaged for statistical analysis (step 30).

2) **ATP-linked respiration** (Basal OCR - DCCD response) is oxygen consumption linked directly to ATP production and is estimated by inhibiting ATP synthase. To calculate, subtract each well’s average DCCD response from its corresponding average basal OCR. Values from across experiments can be averaged for statistical analysis (step 30).

3) **Proton leak** (DCCD response - azide response) is the dissipation of the proton gradient across the inner mitochondrial membrane independent of ATP synthase activity. Calculating proton leak is not straight forward, as we do not have sample wells where both DCCD and azide were injected. To overcome this, we subtract each azide response within an experiment from the corresponding average DCCD response within the same experiment.

Figure 91 shows representative Seahorse data for L4 N2 nematodes.

30. Each mitochondrial parameter should initially be assessed with a one- two- or three-way ANOVA, depending upon how many variables are being assessed (i.e. strain, time, dose etc.). We carry out post-hoc analysis of between-group differences using the Student’s t-test when justified by significant effects observed in our global ANOVAs.
C.2.11 Reagents and Solutions

K-medium

2.36g Potassium chloride (KCl)
3.0g Sodium chloride (NaCl)
1.0L ddH$_2$O

Autoclave to sterilize

Store at room temperature, indefinitely, if stored under sterile conditions

Complete k-medium

150µl 1M Calcium chloride (CaCl$_2$)
150µl 1M Magnesium sulfate (MgSO$_4$)
25µl 10mg/mL Cholesterol (dissolved in 100% ethanol and filter sterilized)

50mL sterile K-medium

Store at room temperature for up to 1 week

Unbuffered EPA water (150)

60mg Magnesium sulfate (MgSO$_4$·7H$_2$O)
60mg Calcium sulfate (CaSO$_4$·2H$_2$O)

4mg Potassium chloride (KCl)

Store at room temperature, indefinitely, if stored under sterile conditions

Sodium hydroxide bleach solution

44mL ddH$_2$O
6mL Clorox Regular Bleach (non-germicidal*, 8.25% sodium hypochlorite)

5 Sodium hydroxide (NaOH) pellets (Avantor Performance Materials, PA)

Store at room temperature for up to 3 days

*It is critical that non-germicidal bleach is used, as some bleaches contain germicides that are toxic to nematodes.

C.3 Commentary

C.3.1 Background Information

Mitochondrial dysfunction has been implicated in myriad human diseases, including cancer (374-376), neurodegeneration (377, 378), and metabolic disorders (379). Thus understanding how toxicant exposure or genetic mutations affect mitochondrial function will be critical in understanding mitochondrial disease. Historically, measurement of oxygen consumption has been a slow process, accomplished by using low-throughput Clarke-type electrode oxygen meters (146). The advent of the Seahorse XF24 and XF96 has not only allowed for rapid and high-throughput determination of basal OCR in in vitro models, but also allows for the determination of ATP-linked respiration, maximal OCR, spare respiratory capacity, and proton leak through injection of various inhibitors of the mitochondrial ETC. Due to the dual probe capacity of the XF®24 and XF®96, it is not only possible to measure OCR, but also extracellular acidification rates (ECAR) thus allowing researchers to identify metabolic shifts from OXPHOS to aerobic glycolysis.
Although the Seahorse XF e24 Analyzer offers nematode researchers the ability to measure the fundamental parameters of the mitochondrial ETC in vivo, it does have limitations. For example, ETC inhibitors cannot be injected in tandem, as done in in vitro assays, limiting its throughput. We have previously demonstrated that the magnitude of response to sodium azide is reduced if injected post-FCCP (228); however, it is possible that injecting a different complete respiratory inhibitor (such as cyanide, or rotenone and antimycin A) post-FCCP would prove more effective. This limitation could be partially overcome by adapting the assay to the XF96 (or XF e96), which has previously been used to measure basal respiration in nematodes (170). Another issue with the XF e24 Analyzer is that it lacks a cooling function; thus, the instrument tends to heat (up to ~25–26°C) as it operates. This issue could be overcome by housing the Seahorse Analyzer in a temperature and humidity controlled apparatus or be limited by maintaining the ambient lab temperature at 20°C. Simultaneous OCR and ECAR measurements have successfully demonstrated metabolic shifts from OXPHOS to aerobic glycolysis, otherwise known as the Warburg effect (155), in the context of toxicant exposure in in vitro models (154, 343). However, ECAR measurements appear to have little value in C. elegans, as mitofusin-deficient fzo-1 (tm1133) nematodes, which exhibit elevated intracellular acidification rates due to increased glycolysis (156), have similar ECAR rates as wild-type nematodes (228). Thus we hypothesize that nematodes do not extrude glycolytic by-products (such as lactate) in the same manner as cells in culture. One final
issue with this assay is lack of a highly specific ATP synthase inhibitor. Oligomycin A, a highly specific inhibitor of the Fo subunit (159) fails to penetrate the nematode cuticle in the timeframe of the assay. Thus we optimized this assay for use with the less specific FoF1 inhibitor DCCD (159), which can inhibit additional cellular ATPases that may contribute to changes in oxygen consumption. Interestingly, even in a cuticle deficient background (bus-8 (e2885)), oligomycin fails to inhibit OCR to the same extent as DCCD (228). Thus we recommend researchers carefully consider the use of DCCD and oligomycin A prior to starting experiments, and confirm results via alternative methods, such as ATP assays.

In addition to these logistical limitations, it is important to keep in mind that a whole-organism approach such as this, while permitting analysis of mitochondrial function in the context of normal intercellular signaling, also results in analysis of respiration in all cells at once. Thus, cell-specific differences may be obscured if they are quantitatively minor, occur in a small number of cells, or are offset but directionally opposite changes in other cells.

**C.3.2 Critical Parameters**

Perhaps the most critical parameter in using the Seahorse XF®24 Analyzer to measure mitochondrial respiration in *C. elegans* is choosing the optimal number of nematodes to add to each well of the islet plate. If too few nematodes are added basal OCR may fall below the instrument’s limit of detection (40 pmol O2/min). Likewise, even
if basal OCR is above 40 pmol O$_2$/min, the addition of DCCD or sodium azide may cause OCR to fall below detection limits. Thus we recommend basal OCR rates be in the range of 200-400 pmol O$_2$/min. Conversely, if too many nematodes are added per well, OCR may rise above the analyzer’s upper limit of detection (1400pmol O$_2$/min) when FCCP is injected. However, even if FCCP-induced OCR does not rise above 1400 pmol O$_2$/min, OCR measurements may still be confounded if oxygen levels fall below 100mmHg or if the mix cycle fails to completely re-oxygenate the well (160-170 mmHg) before the next measurement is taken (see Table 10). This protocol has been optimized to work with 75 L4 nematodes per well; however, if mitochondrial mutant strains are being tested, more L4s may be required (we successfully used 150 L4 complex III mutant nematodes (isp-1(qm150)) per well (228)). Thus, measuring mitochondrial respiration in younger (L1-L3) or older nematodes will require more or less nematodes, respectively, to be loaded per well. As a general guide, we have successfully measured basal and maximal OCR using 35 eight day old wild-type (N2) nematodes and all mitochondrial parameters using 50 four day old germline-deficient (glp-1 (jk1107)) worms; however, we recommend optimizing the number of nematodes added per well, such that basal OCR is in the range of 200-400 pmol O$_2$/min, prior to injection of ETC inhibitors.
C.3.3 Troubleshooting

Table 10 highlights some of the common problems encountered in Seahorse XF24 analysis of mitochondrial function in *C. elegans* and provides potential causes and solutions to these problems.

C.3.4 Anticipated Results

**Basal OCR.** Basal OCR is measured in every well over the first 8 measurements of the analyzer’s run cycle. Typically, the initial 4 OCR measurements are highly variable, but become less variable for the final 4 measurements. This however is not always the case, which is why we recommend running each experiment at least 3 times, separate in time.

**DCCD-response.** Nematodes do not respond instantly to DCCD. Typically OCR falls and plateaus by the ninth OCR measurement post-DCCD injection. Thus, the final 6 OCR measurements are averaged for each well’s DCCD-response. See trouble shooting section (Table 10) if OCR fails to decrease and plateau in response to DCCD.

**FCCP-response.** Nematodes do not respond instantly to FCCP. OCR typically begins to rise by the second OCR measurement post-FCCP injection and should plateau by the fourth measurement. Thus, the final 5 OCR measurements are averaged for a well’s FCCP-response. See trouble shooting section (Table 10) if OCR fails to increase and plateau in response to FCCP.
**Sodium azide-response.** Nematodes respond essentially instantly to sodium azide. OCR will fall and plateau by the time the first OCR is measured post-azide injection, we have never observed differently and recommend making a fresh 80mM sodium azide stock if OCR does not fall and plateau instantly after azide injection.

### C.3.5 Time Considerations

It will take 3-4 days from hypochlorite treatment of gravid adult nematodes to measuring mitochondrial function in the Seahorse Analyzer. This, however, will depend upon the life stage, strain and/or exposure length of the nematodes being used in the study. For the purposes of this protocol, once a synchronous population of L4 nematodes is generated, it will take approximately 10 hours to analyze the nematodes’ response to FCCP, sodium azide and DCCD. On the day of Seahorse XF24 analysis it will take approximately 1 hour to prepare ETC inhibitors for injection, set up the experiment in the WAVE software and add nematodes to the islet plate. The length of Seahorse analysis varies depending upon which drug is being injected, as the number of measurements varies for each drug. For example, analysis of nematodes response to FCCP, sodium azide, or DCCD takes approximately 2.5, 2, or 3.5 hours, respectively. We typically run our FCCP experiments first, followed by sodium azide, and finally DCCD. As analysis of the DCCD response takes the longest, the assay can be left to completion overnight. Typically, when 1 hour remains in a run we begin loading the next ETC inhibitor into a new hydrated sensor cartridge and load nematodes into a new islet
plate. This way, when one Seahorse analysis finishes then next can be started immediately.

**C.4 Acknowledgements**

We would like to thank Dr. Beverly Dance, Dr. Philip Morgan, and Dr. Margaret Sedensky (Seattle Children’s Research Institute, Center for Developmental Therapeutics, Seattle, WA) for generously sharing their protocol for measuring basal OCR in the Seahorse XF24 Analyzer. We would also like to thank Dr. Kevin Bittman (Field Applications Scientist, Seahorse Biosciences, Inc.) for his assistance in adapting the Seahorse XF-24 for use with *C. elegans*. This work was supported by the National Institute of Environmental Health Sciences (R01-ES017540-01A2 to J.N.M.) and the Superfund Basic Research Program (P42 ES0101356-10A2 to J.N.M.).
Table 9: Preparation of ETC Inhibitors for Seahorse XF<sup>e</sup> Analysis.

<table>
<thead>
<tr>
<th>Drug (ETC target)</th>
<th>Stock Concentration</th>
<th>Sensor Cartridge Port Concentration (8x)</th>
<th>Final Well Concentration (1x)</th>
<th>OCR Measurements Post Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCCP <em>(mitochondrial uncoupler)</em></td>
<td>1.25mM dissolved in 100% DMSO (store at -20°C in 350µl aliquots)</td>
<td>200µM dissolved in 16% DMSO</td>
<td>25µM in 2% DMSO</td>
<td>8</td>
</tr>
<tr>
<td>DCCD <em>(ATP synthase inhibitor)</em></td>
<td>2mM dissolved in 100% DMSO (store at -20°C in 200µl aliquots)</td>
<td>160µM Dissolved in 8% DMSO</td>
<td>20µM in 1% DMSO</td>
<td>14</td>
</tr>
<tr>
<td>Sodium Azide <em>(cytochrome c oxidase inhibitor)</em></td>
<td>80mM dissolved in 100% unbuffered EPA water (store at 4°C)</td>
<td>80mM Dissolved in 100% unbuffered EPA water</td>
<td>10mM in 100% unbuffered EPA water</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 10: Troubleshooting Guide for Seahorse XF<sup>e</sup> Analysis in *C. elegans*.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worms do not respond to FCCP and/or FCCP response fails to plateau</td>
<td>FCCP precipitated out of solution</td>
<td>Be sure frozen 1.25mM FCCP stocks are warmed completely to room temperature prior to diluting in EPA water</td>
</tr>
<tr>
<td></td>
<td>25uM FCCP is not efficiently uncoupling respiration</td>
<td>Test a higher concentration of FCCP</td>
</tr>
<tr>
<td></td>
<td>Drug was loaded into wrong injection port</td>
<td>Check the injection ports to make sure the drug was injected and/or was not added to the wrong injection port</td>
</tr>
<tr>
<td>Worms do not respond to DCCD and/or DCCD response fails to plateau</td>
<td>DCCD precipitated out of solution</td>
<td>Be sure frozen 2.0mM DCCD stocks are warmed completely to room temperature prior to diluting in EPA water</td>
</tr>
<tr>
<td></td>
<td>Drug was loaded into wrong injection port</td>
<td>Check the injection ports to make sure the drug was injected and/or was not added to the wrong injection port</td>
</tr>
<tr>
<td>Drug response is outside of the analyzer’s range of detection (i.e. 40-1400 pmol/min)</td>
<td>Too few or too many nematodes per well</td>
<td>Adjust the number of nematodes added per well</td>
</tr>
<tr>
<td>Oxygen levels fall below 100mm Hg during OCR measurements</td>
<td>Too many nematodes loaded per well</td>
<td>Reduce the number of nematodes per well</td>
</tr>
<tr>
<td></td>
<td>Mix cycle is not long enough</td>
<td>Increase the length of the mix cycle (or decrease the length of the measure cycle) to avoid subjecting the worms to anoxic conditions</td>
</tr>
<tr>
<td>Seahorse XFe24 Analyzer is overheating</td>
<td>Ambient lab temperature is not cool enough</td>
<td>Lower the laboratory thermostat (20°C is usually sufficient)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Open the Seahorse tray, turn off the analyzer, and let the instrument sit idle for 30-60 minutes in between assays, giving it time to cool</td>
</tr>
<tr>
<td></td>
<td></td>
<td>House the Seahorse analyzer in a temperature and humidity controlled apparatus</td>
</tr>
</tbody>
</table>
Figure 91: Seahorse XF*24 Analyzer output data for L4 N2 *C. elegans*.

Fundamental parameters (basal OCR, ATP-linked respiration, maximal OCR, spare respiratory capacity, and proton leak) of the mitochondrial ETC, shown for L4 wild-type nematodes.
Appendix D - In vivo Determination of Mitochondrial Function using Luciferase-Expressing Caenorhabditis elegans: Contribution of Oxidative Phosphorylation, Glycolysis, and Fatty Acid Oxidation to Toxicant-Induced dysfunction.

This chapter was published under the same title in *Current Protocols in Toxicology* in 2016 (PMID: 27479364). The authors are Anthony L. Luz, Cristina Lagido, Matthew D. Hirschey, and Joel N. Meyer.

**D.1 Introduction**

Mitochondria are best known for the role they play in ATP production via oxidative phosphorylation; however, mitochondria also play crucial roles in apoptosis (125), calcium homeostasis (381) and retrograde signaling (126), thus playing diverse roles in cellular and organismal health. Mitochondrial dysfunction is causative and/or associated with numerous human diseases, including cancer (375, 382, 383), metabolic syndrome (384), and various neurological disorders (377, 378). Furthermore, growing evidence has demonstrated that mitochondria are an important target of many drugs (e.g. antibiotics and nucleoside reverse transcriptase inhibitors) (91, 299, 385) and environmental toxicants (e.g. polycyclic aromatic hydrocarbons and pesticides) (66, 88, 386), and toxicant-induced mitochondrial dysfunction has been implicated in many diseases, including cancer and neurodegeneration (88, 343, 344).

Because mitochondrial function is dependent upon cellular context and environmental cues (35, 135), it is critical to develop assays capable of rapidly assessing
mitochondrial function, *in vivo*, following toxicant exposure. A short lifecycle (2-3 weeks), high reproductive rate (~300 offspring per gravid adult), and highly conserved mitochondrial biology (112) and biochemical pathways (290) contribute to the utility of the model organism *Caenorhabditis elegans* for studying toxicant-induced mitochondrial dysfunction. Furthermore, significant overlap between the activities of Toxcast phase I and II libraries have recently been described between nematodes and zebrafish, further validating *C. elegans* as an important non-mammalian model (387). Currently, mitochondrial respiration in *C. elegans* can be measured via low-throughput Clark type electrodes (146), or with the higher-throughput, but more expensive Seahorse XF Bioanalyzer (228). Additionally, small metabolites, such as ATP, pyruvate, or NADH can be extracted from nematodes and used to assess mitochondrial health (144, 349); however, this is a time consuming process. Alternatively, transgenic, firefly luciferase-expressing PE255 nematodes can be used to rapidly assess steady-state ATP levels *in vivo* (145, 388). This transgenic model has proven valuable to environmental toxicologists, and has been used to study the effects of heavy metals and 3,5-dichlorophenol (388, 389), 5-fluoro-2-deoxyuridine (326), sewage sludge extract (390), the tobacco-specific nitrosamine 4-(methylNitrosamino)-1-(3-pyridyl)-1-butanone (391), and ultraviolet C radiation (76, 77, 153) on steady-state ATP-levels, and has more recently been used to track nematode development (392), and screen drug-libraries (291).
Here, using PE255 luciferase-expressing nematodes and well-established inhibitors of the mitochondrial electron transport chain (ETC), glycolysis, and fatty acid oxidation (FAO) we describe a novel method that can be used to rapidly screen for alterations in mitochondrial energy metabolism following drug or toxicant exposure. Short-term incubation with these inhibitors depletes steady-state ATP levels. Thus, differential depletion of ATP in toxicant exposed nematodes in response to inhibitors indicates the relative contribution of the targeted cellular process to energy metabolism. Using this approach we recently demonstrated induction of a Warburg-like effect in arsenite exposed PE255 glp-4 nematodes (245), which was confirmed via Seahorse XF and small metabolite analysis, thus further demonstrating this protocol’s utility in detecting toxicant-induced mitochondrial dysfunction.

**D.2 Basic Protocol**

**D.2.1 Luciferase-based in vivo assessment of mitochondrial energy metabolism in C. elegans**

Here, using the PE255 ATP reporter strain (145, 291), and the well-known pharmacological inhibitors rotenone (complex I), thenoyltrifluoroacetone (TTFA, complex II), antimycin A (complex III), sodium azide (complex IV), dicyclohexylcarbodiimide (DCCD, ATP synthase), carbonyl cyanide-p-trifluoromethoxyphenylhydrazon (FCCP, mitochondrial uncoupler), perhexiline (fatty acid oxidation (FAO)), and 2-deoxy-D-glucose (2-DG, glycolysis)) we outline how to rapidly assess mitochondrial energy metabolism following toxicant exposure. All of
these inhibitors have previously been demonstrated to work in *C. elegans* (228, 393-395).

Short-term (1.0 or 4.5 hour) incubation with inhibitors results in changes in steady-state ATP levels. Thus, altered function at the site of inhibition is detected through differential depletion of steady-state ATP levels. For example, the magnitude of ATP depletion following inhibition of ETC complex I with rotenone will be less than in toxicant exposed relative to unexposed nematodes if toxicant exposure has the effect of reducing complex I activity. This is because complex I is already contributing less to maintenance of steady-state ATP levels. Alternatively, the magnitude of ATP depletion will be greater in toxicant exposed nematodes if toxicant exposure is increasing activity of complex I. Figure 92 details the main principles of this assay.

**D.2.2 Reagents**

- OP50 seeded K-agar plates

- Synchronous populations of L1 PE255 nematodes (Transgenic (PE255) N2 (wild type) and PE327 glp-4 (bn2) nematodes available through the *Caenorhabditis* Genetics Center, University of Minnesota)

- K-medium (see recipe)

- Inhibitor stocks (Table 11 outlines all required inhibitors, as well as storage conditions)

- Dimethylsulfoxide (DMSO)

- Unbuffered EPA H₂O (see recipe)
-0.1% (v/v) Triton X-100 (diluted in ddH₂O; store at room temperature indefinitely)

-Glass microscope slides

-Disposable reagent reservoirs

-Multi-channel pipette (capable of pipetting 20-200µl)

-White 96-well plates without lids

-Luminescence buffer (see recipe)

**D.2.3 Equipment**

-Incubator (capable of maintaining temperatures in the range of 15-25°C)

-Centrifuge (e.g. Beckman Coulter equipped for 15mL tubes)

-Dissecting light microscope

-Horizontal vortexer (e.g. Eppendorf MixMate PCR 96)

-Orbital shaker

-Microplate reader (FLUOstar OPTIMA, BMG Labtech) equipped with luminescence optic, 502nm emissions filter, and 485 nm excitation filter

**D.2.4 Nematode Culturing**

Nematodes are cultured on k-agar plates seeded with *E. coli* strain OP50 as previously described (149).
1. Using a sterile Pasteur pipet transfer age-synchronous L1 PE255 nematodes, obtained from sodium hydroxide bleach treatment, to an OP50 seeded k-agar plate. Culture the nematodes until the appropriate life stage for toxicant or drug exposure is reached.

Both PE255 glp4 (strain PE327) and PE255 N2 (wild type) nematodes are available for purchase for a nominal fee, through the National Institutes of Health-supported Caenorhabditis Genetics Center (CGC, University of Minnesota).

This assay was originally developed using germline-deficient, PE255 glp-4 (bn2) nematodes, which are maintained at the permissive temperature of 15°C. Shifting glp-4 nematodes to the restrictive temperature, 25°C, results in sterile, germ cell free nematodes (292). However, we have successfully used most of the concentrations of inhibitors outlined in this assay (see Table 11) with both L4 and 8 day old adult PE255 N2 nematodes, which are maintained at 20°C.

**D.2.5 Toxicant or Drug Exposure**

This assay can be used to assess mitochondrial function following toxicant or drug exposure. The precise length of exposure is at the discretion of the experimenter. However, if using PE255 N2 nematodes we recommend assaying prior to, or after the reproduction period, as reproduction will add variability to experiments. Nematodes can be exposed in liquid or on agar; however, liquid exposures can facilitate drug uptake in nematodes (293). Finally, be sure to thoroughly rinse toxicant exposed
nematodes 3-4 times with 15ml k-medium to remove excess toxicant prior performing
this assay.

**D.2.6 Preparation of Inhibitors**

2. Prepare stocks of 2mM rotenone, 100mM TTFA, 15mM antimycin A, 2mM
sodium azide, 2mM DCCD, 2.5mM FCCP, 10mM perhexiline, and 400mM 2-DG
in either 100% unbuffered EPA water or DMSO as outlined in Table 11. To
minimize freeze/thawing, stocks can be stored in 30µl aliquots at either 4°C or -
20°C (see Table 11).

Titrations of each drug were performed in sterile young adult (cultured on agar
for 72h at 25°C) PE255 glp-4 nematodes (Figure 93 - Figure 100). Concentrations of
each ETC inhibitor that result in a 40-60% depletion of ATP after a one hour exposure
were then chosen.

A 4.5 hour exposure to 50mM 2-DG gave the most consistent reduction in
luminescence in the context of arsenite exposure (245), thus was chosen for all future
experiments.

A 1 hour exposure to 100µM perhexiline increased nematode luminescence
(~25%) in PE255 glp-4-deficient nematodes (Figure 100), and thus was chosen for all
future experiments. Our rationale for this result is detailed in the Anticipated Results,
perhexiline section.
3. Dilute inhibitor stocks with unbuffered EPA H$_2$O to the appropriate 8X working concentrations as outlined in Table 11.

   All inhibitors are dissolved in either DMSO or unbuffered EPA water. 8X working stocks contain zero or 8% DMSO, such that when 12.5µl of the 8X working stock is pipetted into a well of a white 96-well plate (containing 50 nematodes in 87.5µl unbuffered EPA H$_2$O) the inhibitor is diluted to its final, 1X, working concentration in 1% DMSO. A one hour exposure to 1% DMSO does not significantly affect ATP levels in young adult PE255 glp-4, or L4 or 8 day old PE255 N2 nematodes (Figure 93, Figure 101).

D.2.7 Preparation of nematodes for inhibitor exposure

4. Remove PE255 nematodes from the incubator. If nematodes are being exposed to a drug or toxicant (either on agar or in liquid), be sure to rinse the nematodes thoroughly to remove excess toxicant.

   Excess toxicant can be removed by transferring toxicant exposed nematodes to a new 15ml centrifuge tube, and resuspending them in 15ml unbuffered EPA H$_2$O. Nematodes can then be pelleted by centrifuging at 2200 RCF for 2 minutes at room temperature. The supernatant can then be discarded in accord with your university’s guidelines. This process should then be repeated an additional 2-3 times to ensure toxicant is completely removed through dilution.
5. Resuspend the nematodes to a final concentration of 1.0±0.2 nematodes per microliter in unbuffered EPA H$_2$O.

The minimal acceptable concentration is 0.6 nematodes per microliter, as this concentration results in approximately 50 nematodes per 87.5µl; however, we recommend diluting nematodes to a concentration of 0.8-1.2 nematodes per microliter for all samples to minimize variation.

To estimate the number of nematodes per microliter, trim the tip of a 200µl pipette tip, and pipette 20µl of 0.1% Triton X-100 up and down. The triton prevents worm loss due to sticking. Pipet four 20µl drops of nematodes onto a glass slide and count the number of nematodes per 20µl on a dissecting light microscope. Be sure to use a new, triton rinsed tip for each drop, and invert the centrifuge tube several times between counts to resuspend the nematodes.

6. Calculate the volume required to obtain 50 nematodes.

For example, if your concentration of nematodes is 1.0 nematode per microliter, you will pipette 50µl into each well of the 96-well plate to achieve 50 nematodes per well.

7. Pour 5ml 0.1% Triton X-100, 5ml unbuffered EPA H$_2$O, and the nematode suspension into three separate, new, 25ml disposable reagent reservoirs.

8. Using a 200µl multi-channel pipette, pipet 50 nematodes into the appropriate wells of a white 96-well plate.
Prior to pipetting nematodes into a white 96-well plate trim the pipet tips with scissors to increase each tips circumference, which allows large adult nematodes to be pipetted without injury. Then rinse the pipette tips with 0.1% Triton X-100 by pipetting up and down, which prevents nematode loss due to sticking. Nematodes can then be re-suspended in the reagent reservoir prior to their addition to the 96-well plate by pipetting up and down 3-4 times with the multi-channel pipette. Use new, trimmed, triton-rinsed tips each time you resuspend and transfer nematodes.

Figure 102 illustrates how a 96-well plate may be set up for an experiment containing two experimental groups. For example, each group (i.e. control and toxicant exposed) is pipetted into 4 wells of a 96-well plate for each inhibitor or control (i.e. EPA H_2O or 1% DMSO) used. We recommend setting up 2 plates, one for the one hour inhibitor exposure (rotenone, TTFA, antimycin A, azide, DCCD, FCCP, perhexiline), and one for the 4.5 hour inhibitor exposure (2-DG; not shown, but can be setup in a manner similar to Figure 102. Note that 2-DG does not require a 1% DMSO control, as it is dissolved in EPA H_2O).

9. Using a multi-channel pipette bring the volume in each well to 87.5µl with unbuffered EPA H_2O.

EPA H_2O controls (i.e. nematodes unexposed to inhibitors or DMSO) and blank wells can be brought to a final volume of 100µl with unbuffered EPA H_2O.
10. Using a 20µl pipette, add 12.5µl of each 8X inhibitor (prepared in steps 2-3 and outlined in Table 11) to the appropriate wells. Figure 102 outlines how nematodes can be loaded into a 96 well plate; however, this will vary depending upon the number of exposure groups, and inhibitors chosen for each experiment (outlined in Figure 102).

*It should take no longer than 3-4 minutes to load all of the inhibitors for each plate. This is important, because the inhibitor incubation period is only 60 minutes (for ETC, and FAO inhibitors); thus, a longer loading period will introduce variability into the assay. If necessary, samples can be divided onto multiple 96-well plates to limit the amount of time it takes to load all of the inhibitors. However, be sure to include the appropriate EPA H₂O and DMSO controls, as well as blanks for each plate. The addition of inhibitors should be staggered 15-20 minutes for each plate to avoid overlap on the plate reader.*

*At minimum, two plates will be run. The first plate is designated for one hour inhibitor incubations (rotenone, TTFA, antimycin A, azide, DCCD, FCCP, perhexiline, EPA H₂O and 1% DMSO controls), while the second plate is for the 4.5 hour inhibitor incubations (i.e. 2-DG, EPA H₂O control).*

11. Vortex the white 96-well plate for 10 seconds at 1000 rpm using a horizontal vortexer after the final inhibitor has been added.

*Vortexing will help ensure that inhibitors are mixed and completely in solution.*
12. Place the 96-well plate on an orbital shaker at 20°C for 60 minutes or 4.5 hours depending upon which inhibitors are being tested.

**D.2.8 Measuring steady-state ATP levels**

In the presence of ATP, firefly luciferase catalyzes the oxidation of luciferin to generate light. Thus, steady-state ATP levels can be determined *in vivo* by measuring light output, which is proportional to steady-state ATP levels in PE255 nematodes (145). Nematode luminescence can be measured using a microplate reader equipped with a luminescence optic and filters capable of measuring GFP fluorescence (502nm emissions, 485nm excitation). Below we detail how to measure ATP in PE255 nematodes using a FLUOstar OPTIMA (BMG LABTECH) plate reader; however, precise instruction will vary depending upon the microplate reader model being used. A recent visual presentation of this assay is also available (Lagido et al., 2015).

13. Prepare the luminescence buffer (see recipe) 15 minutes prior to the end of the incubation period.

*Luminescence buffer can be prepared in 15ml centrifuge tube covered in foil to protect light-sensitive luciferin.*

14. Turn on the plate reader and open the OPTIMA software 15 minutes before the incubation period has ended. Prepare the plate reader for measuring GFP fluorescence.
PE255 nematodes express a firefly luciferase – GFP fusion protein. Thus by normalizing each well’s luminescence reading to GFP, you can account for overall enzyme content, which will help to normalize each well for slight discrepancies in nematode size and overall nematode counts.

15. Under reader configuration, select the fluorescence optic.

16. Select test setup. Click on fluorescence intensity, and make a new program for measuring GFP. Name (i.e. PE255::GFP) and save the program for future use.

Guidelines for preparing the program are outlined below.

   Plate type: (fill in with the appropriate plate brand)

   Optic used: Top

   Excitation filter: 485nm

   Emission filter: 502nm

   Position delay (s): 0.2

   Kinetic window: 1

   Number of cycles: 1

   Measurement start time (s): 0.0

   Number of flashes: 10

17. Open the newly designed program. Under the Layout tab select the appropriate sample containing wells. Click Okay.
18. Approximately 10 minutes before the incubation period has ended, insert the 96-well plate into the microplate reader. Click on measure and select the appropriate protocol for measuring GFP. Name the current run in the pop-up menu.

19. Click on the gain tab and highlight the entire plate. Click gain adjust.

   *Gain adjusting the entire plate will identify the well with the highest GFP fluorescence, which will be used to normalize the entire plate. The raw gain value should be somewhere around 58,000, although this may vary for other microplate reader models.*

20. Click start measurement. When the measurement has finished exit the OPTIMA software. Turn off the plate reader.

   *GFP measurements are automatically saved in the OPTIMA software.*

21. Carefully remove the fluorescence optic from the microplate reader and replace it with the luminescence optic.

   *For more details see the plate reader’s user manual.*

22. Turn on the plate reader and open the OPTIMA software. Click on Reader Configuration and select luminescence optic.

23. Prime the injector (if applicable) for luminescence buffer injection. First, insert the injector needle into a waste container (50ml centrifuge tube covered with foil)
and the tubing into a 50ml centrifuge tube containing 70% ethanol. Next, select the priming function and flush the injector needle with 2ml 70% ethanol, following by 2ml ddH₂O, and finally prime the injector needle with 1.5ml of luminescence buffer.

*If your plate reader is not equipped with an injecting apparatus you can manually pipette the luminescence buffer into your 96-well plate using a multi-channel pipette and then read luminescence 3 minutes later.*

24. Remove the injector needle from the waste container and place it into the machine’s injection port.

25. Select test setup. Click on luminescence intensity, and make a new program for measuring luminescence. Name (i.e. PE255-Luminescence) and save the program for future use. Guidelines for measuring luminescence are outlined below.

---

**Plate type:** (fill in with the appropriate plate brand)

**Optic used:** Top

**Gain:** 3600

**Emission filter:** lens

**Position delay (s):** 0.2

**Shaking width (mm):** 7

**Shaking mode:** orbital
Number of cycles: 2
Cycle time (s): 180
Measurement start time (s): 0.0
Measurement interval time (s): 1.0

**Injector Setup**

Volume (µl): 50
Pump used: 1
Pump speed (µl/s): 420
Pump syringe volume (ml): 0.5
Injection cycle: 1
Injection start time (s): 0.0

26. Open the newly designed program. Under the layout tab select the appropriate sample containing wells.

27. Click on the injection tab. Make sure all sample containing wells are set to have 50µl of luminescence buffer injected. Click Okay.

28. Click on Measure and select the appropriate protocol for measuring luminescence. Name the current run in the pop-up menu. Click start.

*When the luminescence measurement finishes the results automatically save in the OPTMA software.*
29. When finished with the instrument, wash the injector tubing. First, remove the injector needle from the plate reader and place it into the waste container. Place the injector tubing into ddH$_2$O, and rinse the tubing with 3ml ddH$_2$O, followed by 3ml 70% ethanol. Finally, dry the injector tubing by back flushing 3ml into the waste container.

*Back flushing the injector pushes air through the line to dry it out; any remaining ethanol in the line should then evaporate.*

30. Open results in the OPTIMA Software. Blank correct each well’s GFP and luminescence values by subtracting the EPA H$_2$O blank GFP and luminescence values, respectively. An example of how to normalize data is provided in online Supplemental File 1.

*Each well’s blank-corrected luminescence can be divided by the corresponding blank-corrected GFP value. Alternatively, we generate normalization factors for each well by dividing each well’s blank-subtracted GFP value by the average GFP value for the entire plate. Blank-corrected luminescence values can then be divided by the corresponding normalization factor as detailed in online Supplemental File 1.*

31. Assuming normally distributed data (which has been our experience), assess the effects of each drug initially with a one-, two-, or three-way ANOVA, depending upon how many factors you have (i.e. time, strain, dose, etc.). If different
developmental stages are compared, ATP levels may vary enough that logarithmic transformation of the data is necessary to permit comparison of exposure-or strain-related differences across ages. When warranted, post-hoc analysis can be performed.

D.2.9 Reagents and Solutions

**Complete k-medium** (Store at room temp. for up to one week)

- 150µl 1M calcium chloride (CaCl$_2$; sterilized via autoclave)
- 150µl 1M magnesium sulfate (MgSO$_4$; sterilized via autoclave)
- 25µl 10mg/ml cholesterol (dissolve in 100% ethanol and filter sterilize)
- 50mL sterile k-medium

**K-medium** (Store at room temp., indefinitely, under sterile conditions)

- 2.36g Potassium chloride (KCl)
- 3g Sodium chloride (NaCl)
- 1L ddH$_2$O
  
  Autoclave to sterilize

**LB broth** (Store at room temp., indefinitely, under sterile conditions)

- 0.5g Tryptone
- 0.25g Yeast extract
- 0.5g Sodium chloride (NaCl)
- 50mL ddH$_2$O
Autoclave to sterilize

**Luminescence Buffer** (Make fresh daily)

6.925ml 0.2M Na₂PO₄ (store at room temperature)

3.075ml 0.1M Citric acid (store at room temperature)

100µl DMSO

100µl 5% Triton X-100 (diluted in ddH₂O)

100µl 10mM Luciferin salt (dissolved in ddH₂O; store in 100µl aliquots at -20°C; protect from light)

**Sodium hydroxide bleach solution** (Store at room temp. for up to 3 days)

6mL Clorox bleach (non-germicidal, regular bleach*, 8.25% sodium hypochlorite)

5 Sodium hydroxide pellets (NaOH; Avantor Performance Materials)

44mL ddH₂O

Shake until NaOH pellets are completely dissolved

Each pellet weighs ~89mg.

*It is important to use non-germicidal bleach, as some bleaches contain germicides that are toxic to nematodes.

**Unbuffered EPA water** (150)

60mg Magnesium sulfate (MgSO₄ · 7 H₂O)

60mg Calcium sulfate (CaSO₄ · 2 H₂O)
4mg Potassium chloride (KCl)

1L ddH₂O

**D.3 Commentary**

**D.3.1 Background Information**

The protocol described in this unit details how to rapidly assess the contribution of different pathways to steady-state ATP levels following drug or toxicant exposure in the model organism *C. elegans*. As mitochondrial function is dependent upon cellular context (35, 135), this approach offers the advantage of an *in vivo* model, as well as all of the other benefits associated with working with nematodes. For example, simple RNAi gene knockdown technology (142), and/or outbreeding the PE255 transgene into any of thousands of genetically deficient strains (141) could extend this protocol’s utility to include genetic and gene-environment interaction studies.

Although this protocol offers many advantages over other assays capable of assessing mitochondrial respiratory chain health, it also has limitations. This protocol cannot distinguish between direct enzyme inhibition, substrate limitation, or changes in overall enzyme content as causative of observed changes in inhibitor based ATP-depletion. Instead, this protocol offers an economical way to thoroughly assess mitochondrial health that can then be followed up with more targeted assays, such as metabolomics, Seahorse Analysis, and gene or protein expression studies. Another drawback of this protocol is that it does not directly measure changes in steady-state
ATP levels, but instead measures changes bioluminescence generated by the ATP-powered firefly luciferase enzyme. However, targeting of mitochondrial respiratory chain genes by RNAi gave a bioluminescence response that correlated with steady-state ATP levels in PE255 nematodes (145, 291). Finally, differences in ATP depletion between control and exposed nematodes may be due to compensatory increases in ATP production via other routes, such as glycolysis. However, if this is the case then toxicant-exposed nematodes would be expected to be less sensitive to ATP depletion induced by all ETC inhibitors; thus, toxicant-induced changes at only one or two of the ETC complexes is highly suggestive of altered function at the site of inhibition rather than a compensatory increase in ATP production via an alternative route.

In addition to the inhibitors optimized for this protocol, inhibitors of other metabolic pathways could also be used to further assess toxicant-induced mitochondrial dysfunction. For example, dichloroacetate (DCA), a pyruvate dehydrogenase kinase (negative regulator of the Krebs cycle) inhibitor could be used to assess changes in Krebs cycle activity. We have previously tested this inhibitor in the context of arsenite exposure, but observed no significant changes in bioluminescence under any conditions tested (1-6 hour exposure to either 0.5 or 1.0 mM DCA) (245). Likewise, Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), a glutaminase (GLS1) inhibitor, could be used to test for toxicant-induced changes in glutamine metabolism (396). However, not all metabolic inhibitors will inhibit their intended targets in
nematodes. For example, the mitochondrial uncoupler 2,4-dinitrophenol and the ATP synthase inhibitor oligomycin A do not inhibit mitochondrial respiration in nematodes (228). It is probable that the nematode cuticle, a thick collagenous barrier, limits the uptake of these inhibitors, as oligomycin can inhibit mitochondrial respiration in cuticle-deficient (bus-8) nematodes (228); thus, we use the less-specific ATP synthase inhibitor, DCCD, in the current protocol.

Finally, under certain conditions the ETC complex II inhibitor TTFA can also function as a mitochondrial uncoupler. However, 1mM TTFA did not increase mitochondrial respiration (Figure 103), suggesting that TTFA-induced depletion of ATP (Figure 95) is due to complex II inhibition. Nonetheless, we have also optimized malonate, a competitive inhibitor of complex II for the current protocol (397). Exposure to 15mM malonate for one hour reduces ATP by approximately 50% (Figure 104A), and reduces mitochondrial respiration (Figure 105); however, higher concentrations of malonate were inexplicably found to reduce GFP fluorescence (Figure 104B), which under certain conditions may confound results. Thus, we recommend researchers consider the caveats to working with TTFA and malonate prior to performing experiments.

**D.3.2 Critical Parameters**

*Number of nematodes per well.* This protocol has been optimized to work with 50 nematodes per well of a 96-well plate, and has successfully been used with both L4
and 8 day old adult nematodes. Fewer nematodes (25) have been tested, but tend to give variable luminescence values (data not shown). Alternatively, if this assay is to be used with L2 or L3 nematodes, the experimenter will have to load >50 nematodes per well, but must be careful not to overload the wells as this could result in anoxic conditions.

**Age and genetic background of nematodes.** Early and later life stages are widely considered more sensitive to certain exposures. Thus, concentrations of inhibitors may need to be adjusted depending upon the life stage being investigated. In agreement with this, we observed increased sensitivity to ATP depletion with rotenone, antimycin A, and FCCP in 8 day old PE255 N2 nematodes (Figure 106 - Figure 108). In contrast, reduced sensitivity to TTFA was observed in 8 day old PE255 N2 nematodes (Figure 109), while no age related sensitivities were observed for azide, DCCD, or 2-DG (Figure 110 - Figure 112).

Genetic deficiencies can also sensitize (or protect) an organism from toxicity following toxicant exposure. Therefore, if this protocol is adapted to RNAi studies or if the PE255 transgene is crossbred into other genetically-deficient strains, inhibitor concentrations may need to be adjusted. However, we have crossbred the PE255 transgene into mitochondrial fission-deficient nematodes (*drp-1*), and observed similar responses to inhibitors as with PE255 N2 nematodes (data not shown). Interestingly, young adult PE255 *glp-4* nematodes appear less sensitive to TTFA than either L4 or 8 day old PE255 N2 nematodes, as 1mM TTFA reduced luminescence ~50% in *glp-4*
(Figure 95) and 80-99% in N2 nematodes (Figure 109). On the other hand, young adult PE255 glp-4 nematodes appear more sensitive to sodium azide than either L4 or young adult PE255 N2, as 0.25mM azide reduced luminescence ~50% in glp-4-deficient nematodes (Figure 97), but did not have a statistically significant effect on luminescence in PE255 N2 (Figure 110). Thus, concentrations of TTFA (500µM is effective for N2 versus 1000µM for glp-4) and sodium azide (500µM is effective for N2 versus 250µM for glp-4) will need to be adjusted depending upon the genetic background.

**D.3.3 Troubleshooting**

Table 12 highlights some of the common problems encountered with this protocol, indicates potential causes, and outlines potential solutions to these problems.

**D.3.4 Anticipated Results**

**ETC Inhibitors.** Incubation with inhibitors of the mitochondrial ETC (i.e. rotenone, TTFA, antimycin A, sodium azide, DCCD, FCCP) should dramatically (40-60%) reduce nematode luminescence, as these inhibitors directly interfere with ATP production via oxidative phosphorylation. Therefore, if prior toxicant exposure alters the activity of one or more ETC complexes the magnitude of ATP depletion will be significantly altered compared to unexposed nematodes. For example, if toxicant exposure reduces complex I activity the magnitude of ATP depletion following a one-hour incubation with rotenone will be reduced compared to unexposed nematodes.
**2-Deoxy-D-Glucose.** If drug or toxicant exposure increases glycolysis, then incubation with the glycolysis inhibitor 2-DG should reduce nematode luminescence, whereas 2-DG should have little to no effect on untreated nematode bioluminescence because of the relatively small baseline contribution of glycolysis to ATP.

**Perhexiline.** Perhexiline, a prophylactic anti-anginal medication, prevents mitochondrial fatty acid oxidation (FAO) by inhibiting mitochondrial carnitine palmitoyltransferase-1 (CPT-1), thus preventing the transport of long chain fatty acids into mitochondria (398). Inhibition of FAO with perhexiline results in a shift in cardiac metabolism from the utilization of fatty acids to glucose, which is beneficial because glucose oxidation requires less oxygen per unit of ATP generated. Thus, perhexiline increases cardiac efficiency (399, 400). Like cardiac myocytes (401), germline-deficient nematodes elevate fatty acid oxidation (402). Thus, we hypothesized that treatment of PE255 glp-4-deficient nematodes with perhexiline would increase nematode luminescence by increasing the efficiency of ATP production, which our findings support (Figure 100). In the context of toxicant exposure the effect of perhexiline on luminescence may prove more difficult to interpret. We postulate that toxicant exposures that disrupt glucose catabolism may result in increased FAO, in which case perhexiline would be expected to decrease nematode luminescence by inhibiting FAO. This would provide initial evidence for toxicant-induced changes in FAO that can be confirmed with gene expression or metabolomics studies.
D.3.5 Time Considerations

It will take approximately 48 hours to culture a synchronous population of L4 PE255 nematodes; however, the overall duration of nematode culturing will depend upon the desired larval stage when toxicant exposure is to be initiated. Likewise, the length of toxicant exposure will vary from experiment to experiment. However, following toxicant exposure the entire assay can be performed in approximately six hours (1.5 hours to prepare inhibitors, load nematodes into 96-well plates and start inhibitor exposures, and 1 - 4.5 hours of inhibitor exposure).

When performing assays inhibitors must be pipetted into the appropriate wells of the 96-well plate in a timely manner. This is especially important for the 60 minute inhibitor incubation, as delays will introduce variability into the experiment. The addition of all inhibitors should take no more than 3-4 minutes when using a single channel pipette; however, this can be further reduced by using a multi-channel pipette or by splitting samples onto multiple 96-well plates. However, the addition of inhibitors should be staggered by 15-20 minutes if multiple 96-well plates are being run to ensure no overlap on the microplate reader.

Approximately 10-15 minutes prior to the end of the inhibitor exposure the plate reader can be prepped for the pending GFP and luminescence measurements. Depending upon the plate reader, it will take 5-10 minutes to gain adjust and measure GFP per 96-well plate. Thus to avoid extended incubation periods we typically begin
GFP measurements 5-10 minutes before the end of the incubation period. This allows luminescence to be measured immediately following the end of the 1 or 4.5 hour incubation period, which will help limit variability between experiments.

**D.4 Acknowledgements**

As a Duke Cancer Institute member, I acknowledge support from the Duke Cancer Institute as part of the P30 Cancer Center Support Grant (Grant ID: P30 CA014236). This work was also supported by the National Institute of Environmental Health Sciences (R01-ES017540-01A2).
Table 11: Preparation of Inhibitors for Metabolic Inhibition Assay.

<table>
<thead>
<tr>
<th>Inhibitor (Target)</th>
<th>Stock Concentration</th>
<th>Working Concentration (8x)</th>
<th>Final Concentration (1x)</th>
<th>Incubation period (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone (ETC Complex I)</td>
<td>2mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)</td>
<td>160µM Dissolved in 8% DMSO</td>
<td>20µM in 1% DMSO</td>
<td>1</td>
</tr>
<tr>
<td>*TTFA (ETC Complex II)</td>
<td>100mM dissolved in 100% DMSO (store at 4°C in 30µl aliquots)</td>
<td>8mM Dissolved in 8% DMSO</td>
<td>1mM in 1% DMSO</td>
<td>1</td>
</tr>
<tr>
<td>Malonate (ETC Complex II)</td>
<td>120mM dissolved in 100% unbuffered EPA H₂O (store at 4°C in 1mL aliquots)</td>
<td>120mM dissolved in 100% unbuffered EPA H₂O</td>
<td>15mM in 100% unbuffered EPA H₂O</td>
<td>1</td>
</tr>
<tr>
<td>Antimycin A (ETC Complex III)</td>
<td>15mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)</td>
<td>1.2mM Dissolved in 8% DMSO</td>
<td>150µM in 1% DMSO</td>
<td>1</td>
</tr>
<tr>
<td>**Sodium Azide (ETC Complex IV)</td>
<td>2mM dissolved in 100% unbuffered EPA H₂O (store at 4°C in 1mL aliquots)</td>
<td>2mM dissolved in 100% unbuffered EPA H₂O</td>
<td>250µM in 100% unbuffered EPA H₂O</td>
<td>1</td>
</tr>
<tr>
<td>DCCD (ATP synthase)</td>
<td>2mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)</td>
<td>160µM Dissolved in 8% DMSO</td>
<td>20µM in 1% DMSO</td>
<td>1</td>
</tr>
<tr>
<td><strong>FCCP</strong> (Mitochondrial uncoupler)</td>
<td>2.5mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)</td>
<td>200µM Dissolved in 8% DMSO <strong>To make:</strong> Add 24µl 2.5mM FCCP (100% DMSO) to 276µl unbuffered EPA H₂O</td>
<td>25µM in 1% DMSO</td>
<td>1</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Perhexiline</strong> (Fatty acid oxidation)</td>
<td>10mM dissolved in 100% DMSO (store at 4°C in 30µl aliquots)</td>
<td>800µM Dissolved in 8% DMSO <strong>To make:</strong> Add 24µl 10mM Perhexiline (100% DMSO) to 276µl unbuffered EPA H₂O</td>
<td>100µM in 1% DMSO</td>
<td>1</td>
</tr>
<tr>
<td><strong>2-DG</strong> (Glycolysis)</td>
<td>400mM dissolved in unbuffered EPA H₂O (store at 4°C in 30 µl aliquots)</td>
<td>400mM dissolved in unbuffered EPA H₂O</td>
<td>50mM in 100% unbuffered EPA H₂O</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Concentrations of ETC inhibitors listed in Table 11 caused roughly a 40-60% reduction in bioluminescence in young adult PE255 glp-4 deficient nematodes (Figure 94 - Figure 99), and with the exceptions of TTFA and sodium azide, cause similar reductions in both L4 and 8 day old PE255 N2 nematodes.

*500µM TTFA reduces luminescence approximately 50% in PE255 N2 nematodes (data not shown), while 1000µM causes an 80-99% reduction in PE255 N2 bioluminescence (Figure 109).

**250µM Sodium azide has no significant effect on PE255 N2 bioluminescence, while 500µM azide reduces bioluminescence ~50%.

¥ Malonate, a competitive inhibitor of ETC complex II, can be used in place of TTFA at the discretion of the experimenter. Pros and cons of this are discussed in the Background Information section.
Table 12: Troubleshooting Guide for Metabolic Inhibition Assay.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor caused no, or only a minor decrease in luminescence</td>
<td>Concentration of inhibitor is too low.</td>
<td>Increase inhibitor concentration.</td>
</tr>
<tr>
<td></td>
<td>Inhibitor precipitated out of solution</td>
<td>It is imperative that inhibitor stocks equilibrate to room temperature prior to their addition to the white 96-well plates, as the rapid temperature change may cause the inhibitors to precipitate out of solution.</td>
</tr>
<tr>
<td></td>
<td>Luminescence optic not installed properly</td>
<td>Check that the luminescence optic has been installed properly.</td>
</tr>
<tr>
<td></td>
<td>Luciferin was not added to the luminescence buffer.</td>
<td>Re-prepare the luminescence buffer taking care to add luciferin to the buffer.</td>
</tr>
<tr>
<td></td>
<td>Luminescence buffer failed to inject</td>
<td>Ensure that your plate reader’s injector needle has been properly installed and/or that luminescence buffer was properly added prior to measuring luminescence.</td>
</tr>
<tr>
<td>Inhibitor resulted in greater than a 90% loss of luminescence.</td>
<td>Inhibitor concentration is too high.</td>
<td>Decrease the concentration of inhibitor used.</td>
</tr>
</tbody>
</table>
Figure 92: Workflow for assessing toxicant-induced mitochondrial dysfunction in luciferase expressing PE255 C. elegans.
Figure 93: 1% DMSO does not affect luminescence.

One hour exposure to 1% DMSO does not affect luminescence in young adult PE255 glp-4 nematodes compared to nematodes in EPA water control (One way ANOVA, p=0.65). N=4. Bars±SEM.
Figure 94: Rotenone reduces ATP.

One hour exposure to 10, 15, and 20µM rotenone significantly reduced ATP compared to the 1% DMSO control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.0004). 20µM Rotenone was chosen for future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars±SEM.
One hour exposure to 1mM TTFA significantly reduced ATP compared to the 1% DMSO control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.0005). 1mM TTFA was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars±SEM.

Figure 95: TTFA reduces ATP.
One hour exposure to 100 and 150µM antimycin A significantly reduced ATP compared to the 1% DMSO control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.005). 150µM Antimycin A was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=2-4. Bars±SEM.
Figure 97: Sodium azide reduces ATP.

One hour exposure to 0.25, 0.5, 1.0, 2.5mM sodium azide significantly reduced ATP compared to the EPA water control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.001). 0.25mM Sodium azide was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=2-3. Bars±SEM.
One hour exposure to 20 and 30µM DCCD significantly reduced ATP compared to the 1% DMSO control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.0002). 20µM DCCD was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars±SEM.

Figure 98: DCCD reduces ATP.
One hour exposure to 10 and 25µM FCCP significantly reduced ATP compared to the 1% DMSO control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.0008). 25µM FCCP was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars±SEM.
Figure 100: Perhexiline increase ATP.

One hour exposure to 100µM perhexiline significantly increased ATP compared to the 1% DMSO control in young adult PE255 glp-4 nematodes (one way ANOVA, p=0.0061). 100µM Perhexiline was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars±SEM.
Figure 101: DMSO does not affect luminescence.

One hour exposure to 1% DMSO does not affect luminescence in 2 or 8 day old PE255 N2 nematodes compared to EPA water control (two way ANOVA, main effect of time (p=0.0003), but not treatment (p=0.96) or their interaction (p=0.83)). N=3-7. Bars±SEM.
Figure 102: 96-well plate template for inhibitor exposure.
Figure 103: TTFA does not affect mitochondrial respiration.

Exposure to 1mM TTFA did not affect mitochondrial respiration in young adult PE255 glp-4 nematodes (one-way ANOVA, p=0.53). N=7-22. Bars ± SEM.
Figure 104: Malonate reduces ATP.

One hour exposure to malonate significantly reduced (A) ATP levels (one-way ANOVA, p<0.0001) and (B) GFP fluorescence (one way ANOVA, p=0.014) in young adult PE255 glp-4 nematodes. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=3. Bars ± SEM.
Exposure to malonate reduced mitochondrial respiration in young adult PE255 \textit{glp-4} nematodes (one-way ANOVA, p<0.0001). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD) to control. N=4-12. Bars ± SEM.
Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 20µM rotenone than 2 day old nematodes (2-way ANOVA, main effects of time (p=0.006), treatment (p<0.0001), and their interaction (p=0.008)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD). N=3-4. Bars±SEM.
Figure 107: Old age nematodes are more sensitive to antimycin A.

Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 150µM antimycin A than 2 day old nematodes (2-way ANOVA, main effect of treatment (p<0.0001), and time*treatment interaction (p=0.002), but not time (p=0.19)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD). N=3-4. Bars±SEM.
Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 25µM FCCP than 2 day old nematodes (2-way ANOVA, main effects of time (p=0.02), treatment (p<0.0001), and their interaction (p=0.02)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD). N=3-4. Bars±SEM.
Eight day old PE255 N2 nematodes are less sensitive to ATP depletion following a one hour exposure to 1mM TTFA than 2 day old nematodes (2-way ANOVA, main effects of time (p=0.01), treatment (p<0.0001), and their interaction (p=0.008)). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey’s HSD). N=3. Bars±SEM.
Figure 110: Sodium azide in PE255 N2 nematodes.

One hour exposure to 0.25mM sodium azide did not significantly affect ATP levels in 2 or 8 day old PE255 N2 nematodes compared to EPA water control (2 way ANOVA, main effect of time (p<0.0001), but not treatment (p=0.20) or their interaction(p=0.81)). N=3-4. Bars ± SEM.
Figure 111: DCCD reduces ATP in 2 and 8 day old nematodes.

One hour exposure to 20µM DCCD reduced ATP levels in 2 and 8 day old N2 nematodes (two way ANOVA, main effect of treatment (p=0.003), but not time (p=0.33) or their interaction (p=0.38)). N=3-7. Bars±SEM.
Four and a half hours exposure to 50mM 2-DG did not significantly affect ATP levels in 2 or 8 day old PE255 N2 nematodes compared to EPA water control (2 way ANOVA, main effect of time (p<0.0001), but not treatment (p=0.56) or their interaction (p=0.70)). N=3. Bars ± SEM.
Works Cited


IARC. (2004) Some drinking-water disinfectants and contaminants, including arsenic. IARC.


Biography

Anthony Lincoln Luz was born to Joseph and Cathie Luz on October 13, 1988 in Utica, NY. He received his Bachelors of Science degree, cum laude, in Biology from Cornell University in May 2011.

Publications:


In Preparation:

Luz AL, Smith LL, Meyer JN. Deficiencies in mitochondrial dynamics sensitize Caenorhabditis elegans to arsenite and other mitochondrial toxicants by reducing mitochondrial adaptability. Submitted: Human Molecular Genetics


Meyer JN, Leuthner TC, Luz AL. Mitochondrial fusion, fission, mitophagy, and mitochondrial toxicity. In preparation for Toxicology.


Honors & Awards:

Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31), 2016
Duke University Graduate School Travel Award, 2016, 2017
Nicholas School of the Environment Student Travel Award, 2016, 2017