Modulation of the Redox Environment During Sensitive Developmental Periods Impairs Pancreatic Organogenesis in the Zebrafish (Danio rerio)

Haydee Jacobs

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MODULATION OF THE REDOX ENVIRONMENT DURING SENSITIVE DEVELOPMENTAL PERIODS IMPAIRS PANCREATIC ORGANOGENESIS IN THE ZEBRAFISH (*DANIO RERIO*)

A Thesis Presented

by

HAYDEE MARINA JACOBS

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Environmental Health Sciences
MODULATION OF THE REDOX ENVIRONMENT DURING SENSITIVE DEVELOPMENTAL PERIODS IMPAIRS PANCREATIC ORGANOGENESIS IN THE ZEBRAFISH (Danio rerio)

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ABSTRACT

MODULATION OF THE REDOX ENVIRONMENT DURING SENSITIVE DEVELOPMENTAL PERIODS IMPAIRS PANCREATIC ORGANOGENESIS IN THE ZEBRAFISH (DANIO RERIO)

SEPTEMBER 2018

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The developing pancreas has been identified as a potentially sensitive target for oxidative damage caused by environmental toxicant exposure in the developing zebrafish, Danio rerio. The objectives of this study were to 1) investigate the effects of exposure to a common pro-oxidizing toxicant, Mono(2-ethylhexyl) phthalate (MEHP), on pancreatic development, and 2) elucidate redox-sensitive temporal windows of pancreatic organogenesis in the zebrafish. We also investigated the involvement of Nrf2 (Nfe2l2), a transcription factor involved in the oxidative stress response, in toxicant-mediated pancreatic toxicity. In aim one, zebrafish embryos were exposed to 0 or 200 µg/L MEHP beginning at 3 hours post fertilization (hpf) through 168 hpf, and imaged live under a fluorescence microscope to visualize pancreas development from 48-168 hpf. Glutathione (GSH) and cysteine (Cys) redox couples were quantified by HPLC at 72 hpf, and gene expression was investigated at 96 hpf. This study utilized wild type (AB) Tg(ins:GFP), Tg(gcga:GFP) (endocrine islet), and Tg(ptf1a:GFP) (exocrine pancreas) zebrafish strains. We observed that MEHP exposure significantly reduced endocrine islet area and exocrine pancreas length at all timepoints (48, 72, 96, 168 hpf). No significant changes were observed in the redox potential of GSH or Cys, however MEHP exposure
significantly altered expression of GSH-related genes (*gsr, gstp1*), as well as pancreas-specific genes (*insa, sst2, ptf1a*). These data indicate that the developing pancreas is a sensitive target tissue of embryonic exposure to MEHP. In aim two, we exposed transgenic *Tg(ins:GFP)* and *Tg(gcga:GFP)* zebrafish embryos to water, dimethyl sulfoxide (DMSO), N-acetyl cysteine (NAC), sulforophane (SFN), *tert*-butylhydroperoxide (tBOOH), or *tert*-butylhydroquinone (tBHQ) at 24, 48, or 72 hours post fertilization (hpf), and assessed endocrine islet morphology at 96 hpf. We found both chemical-, stage-, and cell-type specific effects of redox modulation on the endocrine pancreas. Pro-oxidant exposures resulted in decreased ß-cell cluster area and an increased frequency of islet variants, while antioxidant exposures significantly increased ß-cell cluster area. These effects were most significant at the 48 hpf exposure timepoint. α-cell cluster area was only affected by prooxidant exposure at 48 hpf. These results indicate that ß-cells are uniquely sensitive to oxidative stress, specifically at 48 hpf.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
</tr>
<tr>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
</tr>
<tr>
<td>x</td>
</tr>
<tr>
<td>CHAPTER</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1.1. Background</td>
</tr>
<tr>
<td>1.2. Thesis Overview</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2. EMBRYONIC EXPOSURE TO MONO(2-ETHYLHEXYL) PHTHALATE IMPAIRS PANCREATIC ORGANOGENESIS IN THE ZEBRAFISH (DANIO RERIO)</td>
</tr>
<tr>
<td>2.1. Introduction</td>
</tr>
<tr>
<td>2.2. Methods</td>
</tr>
<tr>
<td>2.2.1. Chemicals</td>
</tr>
<tr>
<td>2.2.2. Animals</td>
</tr>
<tr>
<td>2.2.3. Chemical Exposures</td>
</tr>
<tr>
<td>2.2.4. Microscopy</td>
</tr>
<tr>
<td>2.2.5. Image Analysis</td>
</tr>
<tr>
<td>2.2.6. Gene Expression</td>
</tr>
<tr>
<td>2.2.7. Redox Analysis</td>
</tr>
<tr>
<td>2.2.5. Statistics</td>
</tr>
<tr>
<td>2.3. Results</td>
</tr>
<tr>
<td>2.3.1. Gross Morphometrics</td>
</tr>
<tr>
<td>2.3.2. Endocrine Islet Morphometrics</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure                                                                                                                    Page

1. Embryonic exposure to 200 µg/L MEHP does not alter gross morphology from 48 to 168 hpf .................................................................16

2. Embryonic MEHP exposure significantly decreases pancreatic islet area compared to controls at 48, 72, 96, and 168 hpf, and significantly increases the incidence of pancreatic islet variants .............................................................18

3. Embryonic MEHP exposure reduced expression of pancreatic hormone genes insa and sst2 ..........................................................................................................................20

4. Embryonic MEHP exposure shortens the ratio of exocrine pancreas to total fish length at 80 and 168hpf .....................................................................................21

5. Embryonic MEHP exposure significantly reduces expression of ptf1a at 96 hpf .................................................................................................................................22

6. Embryonic MEHP exposure significantly reduces expression of gstp1 and gsr at 96 hpf .................................................................................................22

7. Supplemental Figure 1. Embryonic MEHP exposure does not significantly alter expression of gcga, ghrl, or pdx1 at 96hpf .................................................................30

8. Supplemental Figure 2. Embryonic MEHP exposure does not significantly alter expression of try, amy2a, or cortb1 at 96 hpf .........................................................31

9. Supplemental Figure 3. Embryonic MEHP exposure does not significantly alter the redox potential of GSH or Cys at 72 hpf ..........................................................32

10. Supplemental Figure 4. Embryonic MEHP exposure does not significantly alter expression of ggt1, gclc, or gsta1 at 96 hpf .................................................................33

11. Embryonic exposure to MEHP significantly reduced β-cell area and increased the frequency of pancreatic islet variants in wt and Nrf2a^{b318/b318} homozygous mutant fish .........................................................................................................42

12. Exposure to pro-oxidants tBOOH and tBHQ at 24 hpf decreased β-cell cluster area and increased the frequency of pancreatic islet variants at 96 hpf .........................................................................................................................43

13. Exposure to antioxidants and prooxidants at 48 hpf increased and decreased β-cell cluster area, respectively .................................................................45
14. Exposure to SFN and tBHQ at 72 hpf increased and decreased β-cell cluster area, respectively.
CHAPTER 1

INTRODUCTION

1.1. Background

A global increase in the incidence of metabolic disorders over recent decades has prompted exploration into the environmental etiology of these chronic diseases. Despite the characterization of diet and physical activity as the primary risk factors, a nearly twofold increase in diabetes diagnoses was observed between 1980 and 2011 in the United States alone. Diabetes was recently reported to be the seventh leading cause of death, and accounted for approximately $90 billion in reduced productivity in the US in 2017. It is estimated that the economic cost of diabetes rose 26% from 2012-2017 due to increased diabetes incidence (American Diabetes Association, 2018). It is clearly of critical importance to discover the underlying reasons for this unprecedented increase, as it suggests that factors other than genetics and lifestyle choices may additionally contribute to the pathogenesis of adult-onset metabolic diseases. A growing body of evidence supports the notion that early life exposures to environmental contaminants which occur during critical windows of development may disrupt pancreatic organogenesis, and predispose individuals to adult-onset metabolic disorders (Timme-Laragy, Sant and Rousseau 2015, Sant et al. 2017b, Sant et al. 2016b).

The pancreas has been previously identified as a potentially sensitive target of developmental toxicant exposure (Acharya et al. 2010, Timme-Laragy et al. 2015, Sant et al. 2017b). Organized into endocrine and exocrine compartments, the role of the endocrine pancreas is to rapidly sense minute fluctuations in blood glucose levels, and respond by secreting the appropriate hormones to maintain the energy balance. The
endocrine pancreas is comprised of the Islets of Langerhans, clusters of hormone-secreting cells distributed throughout the body of exocrine tissue. Each endocrine islet consists of α-, β-, δ-, and ε-cells responsible for secreting glucagon, insulin, somatostatin, and ghrelin, respectively. Each of these cell types has a particular function in maintaining glucose homeostasis. The exocrine pancreas is responsible for secreting digestive peptides and proteases critical for extraction of energy from food. Because the pancreas as a whole relies on its highly perfused nature to sense and respond to slight changes in blood glucose, it also likely receives a high toxicant load. Furthermore, it has been shown that β-cells are uniquely sensitive to oxidative damage due to an inherently low antioxidant capacity (Acharya et al. 2010, Tiedge et al. 1997). Because the pancreas plays a critical role in the health of an individual throughout the lifecourse, it is necessary to evaluate how early life environmental exposures may impact its structural and functional development.

The zebrafish model is a uniquely valuable model for studying the effects of developmental chemical exposures. While they share many of the same genetic material, signaling pathways, and cellular functions as other vertebrate species, including humans, zebrafish lay transparent eggs which develop externally to the mother, allowing for easy exposure and manipulation during embryogenesis, until late larval stages. The sequencing of the zebrafish genome has provided intricate tools for studying developmental biology and toxicology, as fluorescent transgenic models and mutant strains are continuously generated (Sipes NS 2011, Stegeman JJ 2010). By utilizing the zebrafish model, we are able to visualize the development of the pancreas in vivo in response to toxicant exposure. The zebrafish pancreas shares structural and functional similarities with the
mammalian pancreas, including an identical cellular makeup. Arising from endodermal progenitor cells, the zebrafish pancreas rapidly differentiates into endocrine and exocrine tissues during the first few days post fertilization (dpf), and is fully mature by 7 dpf (Tiso, Moro & Argenton 2009b). Use of an aquatic model is particularly advantageous for studying the effects of developmental toxicant exposures, because the developmental media can be easily manipulated to mimic environmental or maternal conditions. By introducing common environmental contaminants directly into the water in which the fish develop, we can observe organogenesis in response to toxicant exposure in real time in vivo.

Phthalates are of the most widely studied classes of environmental contaminants. Added to polyvinyl chloride-based plastics to impart flexibility, phthalates are ubiquitously distributed in terrestrial and aquatic environments, and have been detected in nearly 100% of biological samples taken from the U.S. population. Perhaps the most commonly studied compound in the phthalates family, di(2-ethylhexyl) phthalate (DEHP) has been associated with numerous health consequences in human populations. Once it has entered the body, DEHP is hydrolyzed to its primary bioactive metabolite, mono(2-ethylhexyl) phthalate by metabolic enzymes such as pancreatic lipase (Daniel & Bratt 1974). Epidemiologically associated with insulin resistance and type 2 diabetes (T2D) (Corbasson et al. 2014, Corbasson et al. 2016), DEHP and MEHP are also known to disrupt redox signaling in cell culture, and induce oxidative stress by depleting available glutathione (GSH) (Wang et al. 2012, Yang et al. 2012). The redox environment has been shown to play a critical role in development, largely hinging on the availability of cellular GSH. A dynamic network of redox-sensitive proteins governs many critical
Developmental pathways, and altered redox signaling may disrupt critical cellular processes such as proliferation, differentiation, and apoptosis (Hansen & Harris 2013, Timme-Laragy et al. 2013).

Little to no research has been done to explore the connections between developmental phthalate exposure, redox signaling, and pancreatic development. With this project, we aim to test the hypothesis that exposure to chemicals which modulate the redox environment during critical windows of development impairs pancreatic organogenesis, resulting in altered pancreatic architecture and reduced functionality.

1.2. Thesis Overview

The objective of this project is to investigate how the redox environment can be altered by exposure to environmental toxicants, and how these exposures disrupt pancreatic organogenesis. Chapter 2 will explore whether embryonic exposure to a common environmental prooxidant, Mono(2-ethylhexyl) phthalate (MEHP), impairs pancreatic organogenesis, and whether this impairment is likely due to oxidative stress induction and altered redox signaling. Chapter 3 will examine the developmental windows in which redox signaling is critical for endocrine pancreas development.

Experiments for Chapter 2 were completed using several transgenic zebrafish lines. Wild type (AB) embryos were used in all redox analysis and gene expression experiments. Tg(gega:GFP) and Tg(ins:GFP) embryos on a wild type (AB) background were used to examine α- and β-cell cluster area, respectively, while exocrine pancreas length and morphology was assessed using a Tg(ptf1a:GFP) line, also on an AB background. Gross morphology was evaluated in each line used for microscopy.
experiments to ensure that no structural malformations in the fish were occurring due to MEHP exposure, and that the effects observed were pancreas-specific. Zebrafish embryos were exposed to 200 µg/L MEHP beginning at 3 hours post fertilization (hpf), and exposures were maintained every 24 h until culmination of the experiment at 168 hpf. Because developing human embryos would not likely be exposed to DEHP in utero, we chose to expose zebrafish to the toxic metabolite MEHP to evaluate how human babies may be affected by similar exposures. Both the endocrine and exocrine pancreata were imaged via fluorescence microscopy; development of the endocrine pancreas was observed at 48, 72, 96, and 168 hpf, and exocrine pancreas extension was observed at 80 and 168 hpf to maximize the potential for visible differences due to exposure. Expression of the principal endocrine and exocrine pancreatic hormone genes, as well as several genes involved in GSH synthesis and recycling was quantified by quantitative real-time qPCR. Redox analysis was performed using HPLC detection of the soluble thiols GSH and Cysteine (Cys), followed by calculation of redox potentials using the Nernst equation.

Chapter 3 experiments utilized Tg(gcga:GFP) and Tg(ins:GFP) zebrafish lines, as well as a Nrf2a^{fh318/fh318} mutant line, which is characterized by impaired function of the Nrf2 protein. Nrf2a wild type (+/+) and mutant fish were exposed to 200 µg/L MEHP following the same exposure paradigm used in Chapter 2, to determine if any differences in pancreatic sensitivity could be detected due to impaired Nrf2 function. Tg(gcga:GFP) and Tg(ins:GFP) were exposed to four different pro- and antioxidant compounds at three different exposure timepoints during development, and imaged for islet morphology at 96 hpf. Embryos from these lines were exposed to N-acetyl cysteine (NAC) (100 µM),
sulforaphane (SFN) (20 µM), tert-butylhydroperoxide (tBOOH) (77.5 µM), tert-butylhydroquinone (tBHQ) (1 µM), water or dimethylsulfoxide (DMSO) (0.01%) control, at either 24, 48, or 72 hpf, to identify sensitive developmental windows of pancreas development. All embryos were imaged at 96 hpf to observe temporal differences in redox modulation.

Together, these experiments aim to discover whether early developmental exposures to compounds which alter the redox environment can disrupt redox signaling and impair pancreatic organogenesis.
CHAPTER 2

EMBRYONIC EXPOSURE TO MONO(2-ETHYLHEXYL) PHTHALATE IMPAIRS PANCREATIC ORGANOGENESIS IN THE ZEBRAFISH (DANIO RERIO)

This work has been published previously with significant contributions by the following authors:


2.1. Introduction

Phthalates are a class of ubiquitous environmental toxicants, several of which are associated with metabolic and reproductive health disorders (Tickner et al. 2001, Corbasson et al. 2016). Added to polyvinylchloride (PVC)-based plastics to increase flexibility, phthalates are not covalently bound and can leach from plastics when exposed to extreme conditions such as heat or UV light (Tickner et al. 2001). Phthalates have been detected in human biological samples, including blood, pancreatic and intestinal epithelial tissues (Lee et al. 2006). In addition, they have been detected in amniotic fluid and fetal cord blood samples, which indicate that phthalates can cross the placental barrier and pose a potential risk to the exposed fetus (Sathyanarayana et al. 2008).

One of the most widely studied phthalates is di(2-ethylhexyl) phthalate (DEHP), found in consumer products including food packaging, nail polish, medical tubing and toys. Human exposure to DEHP typically occurs through ingestion or via medical equipment (Lin et al. 2011). Once it enters the body, DEHP is rapidly hydrolyzed to mono(2-ethylhexyl) phthalate (MEHP), a highly reactive and toxic metabolite (Frederiksen, Skakkebaek & Andersson 2007). One of the enzymes that hydrolyzes
DEHP to MEHP is pancreatic lipase (Daniel & Bratt 1974). In mouse toxicokinetic studies, MEHP has been detected at high levels in pancreatic tissue, more so than in liver, kidney, blood, or placenta (Tomita et al. 1986). These findings suggest the pancreas is an important target tissue of phthalate exposures.

Both DEHP and MEHP are capable of inducing oxidative stress by reducing available glutathione (GSH) and disrupting GSH-related gene expression (Sant et al. 2016a, Wang et al. 2012), and both have been associated with metabolic dysfunction, insulin resistance and type 2 diabetes (Corbasson et al. 2016, Lin et al. 2011). Exposure to MEHP during early developmental windows has been shown to negatively impact development due to induction of oxidative stress, implicated by increased frequencies of neural tube defects and altered male and female genital morphology following MEHP exposure in rodent models (Sant et al. 2016a, Wang et al. 2012, Gray et al. 2000). We have recently shown that developmental exposure to prooxidants negatively impacts pancreatic development and produces structural islet variant morphologies and truncation of the exocrine pancreas, similar to those produced by embryonic MEHP exposure (Sant et al. 2016b). Thus, we hypothesize that disruptions in redox signaling may be a potential mechanism by which MEHP alters pancreatic morphology.

The zebrafish model is widely integrated in developmental biology and toxicology research (Truong et al. 2014, Tiso et al. 2009b, Yang et al. 2009). The availability of pancreas-specific transgenics make it a particularly insightful model in which to investigate toxicological impacts on pancreas development, as the development of both the endocrine and exocrine pancreas can be easily visualized in real-time throughout development in live embryos. In zebrafish, the first pancreatic bud forms
dorsally from the endoderm at the 16-somite stage during organogenesis, governed by expression of the pancreatic and duodenal homeobox gene (pdx1). Between 24 and 48 hpf, the dorsal bud rotates along with the gut and gives rise to the primary endocrine islet, a core of $\beta$-cells surrounded by $\alpha$-cells and $\delta$-cells. A second bud arises anteroventrally during the pharyngula stage around 40 hpf (reviewed in Tiso et al. 2009), and elongates to form the bulk of exocrine tissue between 48 and 72 hpf. The anteroventral bud differentiates to form the pancreatic ducts, governed by dual expression of pdx1 and the pancreas-specific transcription factor alpha (ptf1a) (Tiso et al. 2009). Importantly, these developmental signaling pathways and processes of pancreatic organogenesis are highly conserved among vertebrates, including zebrafish (Lele & Krone 1996). Additional advantages of the zebrafish model include large clutches of transparent embryos that develop rapidly, externally to the mother. These attributes make the zebrafish embryo one of the best-suited models for investigating pancreatic development in vivo.

We recently reported that prooxidant compounds and MEHP have the ability to produce similar structural pancreatic anomalies, including hypomorphic beta cell clusters in primary islets of zebrafish embryos (Sant et al. 2016b). Here, we aim to characterize the observed effects of embryonic exposure to MEHP on endocrine and exocrine pancreatic organogenesis, identify the pancreas as a sensitive target tissue of developmental exposure to MEHP, and provide the groundwork to investigate the functional toxicological impacts of MEHP on developing pancreas in the zebrafish embryo model.
2.2. Materials and Methods

2.2.1. Chemicals

All chemicals used in these experiments were of the highest purity grade available. Mono(2-ethylhexyl) phthalate (MEHP) was purchased from AccuStandard (New Haven, CT, USA). Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburg, PA, USA). A stock solution of 2 mg/mL was prepared by diluting neat MEHP in DMSO. Solutions were stored in amber vials at -20 ºC and vortexed before each use. All procedures involving the handling of MEHP were performed utilizing proper PPE and standard laboratory safety precautions. All other chemicals used in this study were purchased from Fisher Scientific.

2.2.2. Animals

Adult zebrafish were maintained in accordance with the guidelines laid out in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and with authorization from the University of Massachusetts Amherst Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3551-01). Fish were maintained in a recirculating zebrafish system (Aquaneering, San Diego, CA, USA) at 28.5 ºC on a daily 14 h light:10 h dark cycle, and fed once daily with the recommended amount of Gemma Micro 300 (Skretting; Westbrook, ME, USA). Breeding fish populations were maintained in tanks containing an approximate 1:2 ratio of males to females. Zebrafish embryos were collected daily from tanks approximately 0-1 hours post fertilization (hpf), washed thoroughly, and placed in 100 mm polystyrene petri dishes containing 0.3X Danieau’s solution (Westerfield 2007).
Wildtype AB zebrafish were obtained from Boston Children’s Hospital (Boston, MA) and were used in the redox and gene expression experiments. Transgenic zebrafish strains (on AB background) were obtained from Dr. Philip diIorio at the University of Massachusetts Medical School (Worcester, MA, USA), and Dr. Jennifer Moss at Duke University. The Tg(ins:GFP) and Tg(gcga:GFP) strains, which co-express green fluorescent protein (GFP) with insulin and glucagon expression respectively, were used to visualize pancreatic islet development (diIorio et al. 2002b, Pauls et al. 2007b). A Tg(ptf1a:GFP) strain was used to visualize the development of the exocrine pancreas (Lin et al. 2004).

2.2.3. Chemical Exposures

Zebrafish embryos were exposed to MEHP beginning at the mid-blastula transition (3 hpf). Embryos were transferred to 20 mL glass scintillation vials containing 10 mL dosing solutions (0.01% DMSO control, 200 µg/L MEHP in 0.3X Danieau’s medium), with 5 embryos per vial. Solutions were refreshed daily through 168 hpf (7 days post fertilization) by completely changing media of both control and exposed groups. Embryos were manually dechorionated at 24 hpf using watchmaker’s forceps under a dissection microscope (Leica Microsystems, IL, USA). The concentration of MEHP was chosen based on other zebrafish studies in the literature which showed 200 µg/L to produce sub-lethal effects (Zhai et al. 2014). Our preliminary exposure studies confirmed that this concentration does not produce gross malformations in developing zebrafish.
2.2.4. Microscopy

To observe larval growth and the development of the primary pancreatic islet, \( Tg(ins:GFP) \) eleutheroembryos and larvae were imaged at 48, 72, 96, and 168 hpf, using an inverted fluorescence microscope with a light cube GFP filter (EVOS FL Auto, Life Technologies, Pittsburgh, PA, USA). Images were captured with 4X, 10X and 20X objectives under transmitted light and GFP filters to assess gross morphology and islet development. To observe the extension of the exocrine pancreas, \( Tg(ptf1a:GFP) \) larvae were imaged at 80 and 168 hpf, previously identified as sensitive timepoints in exocrine pancreas development (Sant et al. 2017b). Larvae were examined using an upright Olympus compound fluorescence microscope equipped with a Zeiss Axiocam 503 camera and Zen analysis software (Zeiss, USA). Images were captured with 4X and 10X objectives under transmitted light and GFP filters to assess total fish length and exocrine pancreas length.

Prior to imaging, eleutheroembryos and larvae were washed in petri dishes containing 30-40 mL 0.3X Danieau’s, briefly anesthetized in 0.3X Danieau’s containing MS-222 (Westerfield 2007), and staged in 3% methylcellulose. The zebrafish pancreas is located on the right side of the body, and fish were oriented laterally to optimize pancreas visualization on either the right or left side depending on whether the inverted or upright microscope was used in the acquisition. After imaging, the fish were washed thoroughly in fresh Danieau’s medium.

2.2.5. Image Analysis

Prior to analysis, images were blinded, and then analyzed using EVOS or Zen (Zeiss) analysis software. To account for acquisition artifact, all images captured on the
inverted EVOS were mirror-flipped prior to analysis using Image J software (downloaded from NIH.gov). Gross morphology and image quality was assessed first to determine whether the images met quality control parameters, including correct staging, exposure, and image acquisition. Gross morphology was assessed by measuring total fish length, yolk sac area, and pericardial sac area. To determine total fish length, images acquired with a 2X objective were used to draw a straight line from the fish’s tail to jaw, and length was calculated by the software. Yolk sac and pericardial sac areas were measured by tracing the perimeter of each structure. To determine the area of the endocrine islets (either β-cells or α-cells), images acquired with a 10X objective captured under a GFP filter were used to trace the perimeter of the cell cluster, and areas were calculated by the software. Exocrine pancreas length was measured after imaging with a 5X objective in the same fashion, by drawing a straight line from the posterior tail of the pancreas to the posterior end of the endocrine islet, visualized as a dark indent in the fluorescent exocrine tissue. Relative exocrine pancreas length was determined by calculating a ratio of exocrine pancreas length to total fish length.

2.2.6. Gene Expression

At 96 hpf, RNA was collected from AB eleutheroembryos to assess gene expression of pancreas glucoregulatory hormones and digestive enzymes, and genes related to glutathione. A total of 6 samples from each exposure group were collected with pools of 7 fish per sample, and stored in RNA Later (Fisher Scientific) at -80 °C until RNA isolation. RNA was isolated using the GeneJET RNA Purification Kit purchased from Fisher Scientific (Waltham, MA, USA), following the included isolation protocol for whole tissue purification. RNA concentrations and sample purity were determined
using a µLITE spectrophotometer purchased from BioDrop (Cambridge, UK). Immediately following RNA quantification, 500 ng RNA were converted to cDNA by reverse transcription using the iScript cDNA Synthesis Kit made by Bio-Rad, and all samples were diluted to 0.25 ng/µL cDNA in nuclease-free water, and stored at -20 ºC until use.

Using a Bio-Rad CFX Connect Real-Time PCR Detection System, Quantitative Real-Time PCR (qRT-PCR) was performed to assess the expression of the pancreas hormone genes and glutathione-related genes. A 20 µL reaction mixture was prepared containing 5 µL nuclease-free water, 5 pM of each primer, and 10 µL 2x iQ SYBR Green Supermix (BioRad), and 4 µL cDNA template. Previously designed and optimized primers for β-actin (actb), β-2-microglobulin (b2m) and preproinsulin a (insa) have been described in published literature (Timme-Laragy et al., 2015). Additional pancreas genes, glucagon (gcga), ghrelin (ghrl), somatostatin 2 (sst2), pdx1, ptf1a, trypsin (try), chymotrypsinogen B1 (crtb1), and α-amylase 2A (amy2a) were investigated using PrimePCR primers purchased from Bio-Rad. Primers for the genes encoding glutathione-cysteine ligase catalytic subunit (gclc), glutathione-S-transferase A1 (gstal), glutathione-S-transferase P (gstp), glutathione-disulfide reductase (gsr), and gamma-glutamyltransferase 1b (ggt1b), have been previously described in published literature (Sant et al. 2017a). Gene expression data were analyzed using Bio-Rad CFX software, and the ΔΔCT method was used to calculate fold-changes (Livak and Schmittgen, 2001). All fold changes were standardized relative to the biweight mean center of β-actin and b2m expression (Dombi 2010).
2.2.7. Redox Analysis

Quantification of glutathione and cysteine redox pairs was performed using high-performance liquid chromatography, as previously described in (Jones 2002, Harris and Hansen 2012) and previously performed in (Sant et al. 2017a, Timme-Laragy et al. 2013). Briefly, five pools of 15-20 embryos were preserved in buffer containing perchlorate, boric acid, and γ-glutamylglutamate and stored at -80°C until use. Samples were derivatized using dansyl chloride, and analyzed using a Waters 2475 fluorescence detector coupled to a Waters 2695 separations module fitted with a Supelcosil LC-NH2 column. Reduced and oxidized glutathione (GSH, GSSG) and cysteine (Cys, CySS) was quantified using Waters Empower software, using excitation and emission wavelengths of 335 and 518 nm, respectively. Flow rate was 1 mL/min, using a gradient method switching between two mobile phases described in (Harris and Hansen 2012). Redox potentials were calculated using the Nernst equation (pH 7.4): \[ E_h = E_0 + \frac{RT}{nF} \log \left( \frac{[GSSG]}{[GSH]^2} \right), \]
where \( E_0 = -264 \text{ mV} \) and \( \frac{RT}{nF} = 30 \).

2.2.8. Statistics

All microscopy experiments were repeated in triplicate. Statistical significance was determined by Students unpaired t-tests, chi-squared tests, or ANOVA with a Fishers post hoc test. Gene expression was analyzed using the \( \Delta \Delta C_T \) method (Livak K. J. 2001). Fold change calculations were normalized to the biweight mean center of two housekeeping genes, \( \text{bactin} \) and \( \text{b2m} \) expression, and the DMSO controls. Data are presented as the mean plus or minus the standard error of the mean (SEM).
2.3. Results

2.3.1. Gross Morphometrics

Developmental exposures to toxicants including MEHP have the ability to alter gross morphology. However, we selected a dosing concentration that is both environmentally relevant, and has been shown to be below embryotoxicity thresholds in previous zebrafish studies (Swan 2008, Sant et al. 2016a, Zhai et al. 2014). To determine if any pancreas variants seen were associated with gross malformations in the fish, total fish length, yolk sac area, and pericardial sac area were measured to assess MEHP effects on growth, yolk utilization, or cardiovascular health. No significant changes in length, yolk sac area, or pericardial sac area were observed due to MEHP exposure at this concentration (200 µg/L) (Fig. 1A-1D).

Figure 1. Embryonic exposure to 200 µg/L MEHP does not alter gross morphology from 48 to 168 hpf. A) Length (rostral to caudal), B) yolk sac area, and C) pericardial sac area were measured at 48, 72, 96, and 168 hpf using Zen analysis software with a 2x objective. No significant differences in length, yolk sac area, pericardial area, or gross deformities were detected between exposed or control eleutheroembryos and larvae at any time point examined (n = 30 fish; p>0.05).
2.3.2. Endocrine Islet Morphometrics

The Islets of Langerhans are essential to maintain glucose homeostasis, as they contain the only cells capable of secreting insulin and glucagon. It has been well-established that a decrease or loss of endocrine islets, especially the β-cells, is a common characteristic of both type 1 and 2 diabetes (Akirav, Kushner and Herold 2008, Matveyenko and Butler 2008). To assess whether early embryonic MEHP exposure alters the growth of the endocrine islet during development, Tg(ins:GFP) and Tg(gcga:GFP) zebrafish were used to visualize the area of the insulin-producing β-cells and glucagon-producing α-cells, respectively.

Following subchronic MEHP exposure, β-cell cluster area was quantified by fluorescence microscopy using Tg(ins:GFP) embryos, eleutheroembryos, and larvae at 48, 72, 96, and 168 hpf. We found that β-cell cluster area was significantly decreased in exposed fish compared to controls at all timepoints, most significantly at 48 hpf (p<0.001) where there was a 17.6% reduction in area (Fig. 2A). Similarly, α-cell area was assessed using Tg(gcga:GFP) embryos and eleutheroembryos at 48, 72, 96, and 168 hpf, using fluorescence microscopy. At each timepoint observed, α-cell area was also decreased in MEHP exposed groups compared to controls, most significantly at 96 and 168 hpf, with 27% and 32.38% reductions in α-cell area, respectively (p<0.001) (Fig. 2B). The β-cell cluster is surrounded by the glucagon-producing α-cells; together, these data demonstrate that developmental exposure to MEHP results in hypomorphic islets.

In addition to measuring islet area, Tg(ins:GFP) and Tg(gcga:GFP) zebrafish were used to assess islet morphology. While the islet normally develops in a compact, spherical cluster of endocrine cells, several variant morphologies have been previously...
described following toxicant exposures using the Tg(ins:GFP) strain, including
fragmented, stunted, and hollow islets, as well as ectopic β-cells (Sant et al. 2016b, Sant et al. 2017c). To observe if MEHP exposure results in any of these variants in addition to

Figure 2. Embryonic MEHP exposure significantly decreases pancreatic islet area compared to controls at 48, 72, 96, and 168 hpf, and significantly increases the incidence of pancreatic islet variants. A) Pancreatic β-cell cluster area was visualized using Tg(ins:GFP) zebrafish eleutheroembryos and larvae following treatment with MEHP or DMSO, and islet area was measured using Zen analysis software. B) Pancreatic α-cell cluster area was visualized in Tg(gcga:GFP) zebrafish eleutheroembryos and larvae following treatment with MEHP or DMSO and measured using Zen analysis software. Islet architecture was assessed using Tg(ins:GFP) zebrafish eleutheroembryos and larvae. Morphologies that deviated from the normal spherical islet shape were categorized as fragmented, stunted, hollow, or having ectopic b-cells. Variants were quantified across three study replicates. C) The position of the endocrine islet is shown on a 96 hpf eleutheroembryo, as well as representative images of the islet variants observed. D) Pie charts show the distribution of each variant, and numbers shown are the percentage of normal islets at each timepoint (n = 40 for 48, 72, 96; n = 20 for 168 hpf; *p < .05; **p < .005).
the overall decrease in islet area, islet morphology was assessed at 48, 72, 96, and 168 hpf. At all timepoints observed, MEHP exposure increased the frequency of pancreatic islet morphology variants compared to controls when β-cell area was assessed (Fig. 2D). No islet variants were observed when α-cell area was assessed. While these variants were occasionally observed among controls, a significantly greater frequency of total variant islet morphologies was seen in MEHP-exposed groups (p<0.05).

2.3.3. Endocrine Hormone Gene Expression

To address whether the MEHP-induced reduction in islet area resulted in changes to gene expression, expression of several endocrine hormone genes was quantified at 96 hpf by qPCR and analyzed using the ∆∆CT method, normalized to two housekeeping genes, β-actin and beta-2-microglobulin (b2m). Responsible for stimulating cellular uptake of glucose, Insulin (encoded by preproinsulin a; insa) is secreted by the β-cells of the endocrine islet. In contrast to insulin action, the α-cells produce Glucagon (encoded by glucagon a; gcga), which acts on the liver to break down glycogen and release free glucose into the bloodstream. Secreted by the δ-cells, Somatostatin (encoded by somatostatin 2; sst2) has many signaling responsibilities, including the inhibition of insulin receptor signaling. Ghrelin (encoded by ghrelin; ghrl), thought of as the “hunger hormone”, is produced by the ε-cells of the islet to stimulate food-seeking behavior, and works inversely to Leptin, which is produced by adipocytes. In addition to these endocrine hormones, we examined the expression of Pdx1 (encoded by pdx1), which mediates the expression of the aforementioned glucoregulatory genes (Tiso et al. 2009b). In combination, these genes are responsible for maintaining glucose homeostasis, and disruption of their expression could have significant metabolic consequences.
We found that embryonic MEHP exposure resulted in either a statistically significant downregulation, or trend towards downregulation, in expression of each of the endocrine hormone genes examined (Fig. 3A, 3B; Supp. Fig. 1). Expression of insa and sst2 were reduced by 31.5% and 35.5% compared to controls (p=0.0069 and p=0.0018), respectively (Fig. 3). Expression of gcga, ghrl and pdxl trended towards reduction by 13%, 40.6% and 17.2% (p=0.541, p=0.0617, and p=0.392), respectively (Supp. Fig. 1).

2.3.4. Exocrine Pancreas Morphometrics

While the endocrine islets produce the majority of the glucoregulatory hormones, the body of the pancreas is composed primarily of exocrine tissue, which produces
digestive peptides. The exocrine pancreas extends posteriorly during the 48 to 96 hpf window of zebrafish development. The primary islets appear between 24 and 48 hpf, and are located in the region proximal to the gut, while the secondary islets begin to develop in the distal body and tail of the exocrine pancreas, around 168 hpf, once extension is complete (Tiso et al. 2009b). Congenital shortening of the pancreas has been associated with diabetic phenotypes in human studies (Elayat, el-Naggar and Tahir 1995, Agabi and Akhigbe 2016). Toxicological perturbations have been proposed as a potential mechanism for exocrine pancreas insufficiency, and for this reason we examined whether MEHP exposure may impact the development and extension of the exocrine pancreas.

![Figure 4](image)

**Figure 4.** Embryonic MEHP exposure shortens the ratio of exocrine pancreas to total fish length at 80 and 168 hpf. Exocrine pancreas morphology was assessed using *Tg(ptf1a:GFP)* zebrafish eleutheroembryos and larvae. A) The position of the exocrine pancreas is shown, as well as representative images of shortened pancreata observed. B) Pancreas length was measured using Zen analysis software, from the center of the primary endocrine islet to the tail of the pancreas. Exocrine pancreas length was normalized to total fish length. Data presented are mean ratios of pancreas to fish length ± SEM (n = 30; *p < 0.05).

(Sant et al. 2016b). Exocrine pancreas extension was visualized at 80 and 168 hpf following subchronic MEHP exposure using the *Tg(ptf1aGFP)* transgenic line, and pancreas length was measured from the center of the primary endocrine islet to the posterior pancreatic tail using Zen analysis software. Total fish length was also measured, and pancreas length is reported as a proportion of total length to account for size.
variability. We found a significant overall decrease in the ratio of exocrine pancreas to total fish length in MEHP exposed individuals compared to controls at both timepoints (p<0.05), which suggests that MEHP exposure may contribute to a shortened pancreas phenotype (Fig. 4A, 4B).

2.3.5. Exocrine Hormone Gene Expression

While the endocrine pancreas is responsible for secreting glucoregulatory hormones, the exocrine pancreas also plays a role in maintaining glucose homeostasis by producing digestive peptides. We measured expression of four critical exocrine genes: pancreas-specific transcription factor 1a (ptf1a), the proteases trypsin (try) and chymotrypsinogen B1 (ctrb1), and pancreatic amylase (amy2a). We observed altered expression of each exocrine gene in the exposed group compared to the controls.

Consistent with the shortened pancreas phenotype observed in the Tg(ptf1a:GFP) line, expression of ptf1a in the MEHP exposed group was significantly reduced compared to controls by 33.9% (p=0.023) (Fig. 5). We also observed a trend towards a decreased
expression of *crtb1*, and increased expression of *try* and *amy2a* but this was not statistically significant (Supp. Fig. 2).

2.3.6. Redox Analysis & GSH Gene Expression

MEHP has been reported to cause oxidative stress in numerous studies. Here, we used biochemical, molecular, and genetic approaches to determine whether MEHP caused oxidative stress in embryos, and whether this contributed to the aberrant β-cell development. First, we measured the content of the redox couples GSH:GSSG and Cys:CySS to identify disrupted redox homeostasis in whole embryos. Quantification of these redox couples revealed no significant changes in GSH, Cys, GSSG, CySS, or redox potential ($E_h$) of GSH or Cys (Supp. Fig. 3). $E_h$ (GSSG/GSH) remained constant between exposed and control samples, at -230.656 mV and 229.583 mV, respectively (p=0.634).

![Figure 6](image.png)

**Figure 6.** Embryonic MEHP exposure significantly reduces expression of *gstp1* and *gsr* at 96 hpf. AB zebrafish were exposed to MEHP from 3 to 96 hpf, and gene expression was assessed at 96 hpf. Data are presented as mean fold change normalized to the biweight mean center of *bactin* and *b2m* expression ± SEM (n = 6; *p<0.05).*
Eh CySS/Cys also remained constant between exposed and control samples at -154.68 mV and 155.543 mV, respectively (p=0.741). Total GSH remained unchanged between exposed and control samples, at 3459.544 µM and 3447.519 µM, respectively (p=0.964), while total Cys increased slightly from 1526.006 µM in control samples to 1646.364 µM in exposed samples (p=0.634) (Supp. Fig. 3A-C). While the means of these measures were not significantly different, the variation within the MEHP-treated embryos was much less than the controls (Supp. Fig. 3C).

To expand our investigation into how MEHP exposure may influence the redox environment in the developing embryo, we assessed the expression of selected genes encoding enzymes and subunits involved in GSH synthesis, recycling, and utilization. During oxidative stress conditions, S-glutathionylation of proteins is catalyzed by glutathione S-transferase pi 1 (gstp1) and glutathione S-transferase alpha 1 (gsta1) to stabilize protein structure and aid in cellular detoxification. Availability of GSH is increased by glutathione-disulfide reductase (gsr), which recycles oxidized glutathione-disulfide (GSSG) into reduced GSH. Additional enzymes encoded in part by gamma-glutamyltransferase 1b (ggt1b) and glutamate-cysteine ligase catalytic subunit (gclc) increase cellular cysteine availability and catalyze glutathione synthesis respectively (Sant et al. 2017a). We observed no change in expression of gsta1, ggt1b, and gclc in response to MEHP exposure (Supp. Fig. 3). However, we did find significant alterations in expression of gstp1 and gsr. Gstp1 expression decreased significantly by 20 % (p=0.042), while gsr expression doubled (p=0.0073) (Fig. 6).
2.4. Discussion

Human exposure to MEHP is widespread and poses a health risk to the developing embryo. Epidemiology studies have detected MEHP in nearly 100% of biological samples taken from the U.S. population, and MEHP exposure has been associated with increased risk for cancer, obesity, neurological disorders and reproductive dysfunction in both men and women (Sun et al. 2014, Corbasson et al. 2016, Benjamin et al. 2017). Experimental evidence in humans and animals have demonstrated that MEHP is capable of crossing the placental barrier and exposing the developing embryo (Sathyanarayana et al. 2008).

Previous studies in zebrafish have demonstrated that developmental MEHP exposure results in embryotoxicity and endocrine disruption. At elevated concentrations (250-500 µg/L), embryonic MEHP exposures result in lethality and overt embryo toxicity (Lammer et al. 2009, Kroese et al. 2015). While both terrestrial and aquatic species are rarely exposed to such concentrations, low-dose effects of MEHP exposure in zebrafish have also been characterized. These include thyroid endocrine disruption, exhibited by significantly reduced levels of whole body triiodothyronine (T3) and thyroxine (T4), occurring at concentrations of 200 µg/L (Zhai et al. 2014). Additionally, chronic MEHP exposure induced reproductive dysfunction in both male and female zebrafish by reducing gamete production and quality at concentrations as low as 4 µg/L (Zhu et al. 2016).

In the present study, we show that early embryonic MEHP exposure is capable of disrupting the development of both the endocrine and exocrine pancreas, demonstrated by alterations to pancreatic growth, structure, and gene expression. While non-lethal
pancreas defects often go undiagnosed, the presence of several congenital variants has been associated with increased risk for adult-onset diabetes and pancreatitis. The endocrine pancreas is responsible for the production of essential endocrine hormones that maintain glucose homeostasis, while the enzymes secreted by the exocrine pancreas are critical for digestion and facilitate the breakdown of complex carbohydrates. While uncommon, structural alterations of the endocrine pancreas, such as ectopic endocrine tissue, and exocrine pancreas malformations such as pancreatic agenesis (a shortened pancreas), are associated with type 1 and 2 diabetes (Bento, Baptista & Oliveira 2013).

We observed a significantly elevated frequency of hypomorphic and variant endocrine islet morphologies in response to MEHP exposure. Using Tg(ins:GFP) and Tg(gcga:GFP) transgenic lines to visualize β- and α-cell development, respectively, we observed decreased islet areas which persisted from 48 to 168 hpf. This finding is consistent with rodent studies which have shown that developmental exposure to the parent compound of MEHP, DEHP, disrupts mitochondrial function in β-cells, resulting in reduced β-cell cluster area and architectural variation of the islet, effects which persist into adulthood and disrupted glucose metabolism throughout the life course (Lin et al. 2011). Additionally, we show an elevated frequency of variants in islet morphology. Several of these islet variants are similar to those found in humans, such as ectopic pancreatic tissue, which occurs in approximately 10% of the human population (Bento et al. 2013). In addition to ectopic tissue, we have shown an increased frequency of “hollow” β-cell clusters, which appear as a circular ring of fluorescent cells. This “hollow islet” morphology phenocopies islet morphology identified in pdx1 zebrafish morphants (knockdown by antisense morpholino oligonucleotides), which suggests that MEHP may
be disrupting \textit{pdx1} function to produce this phenotype (Kimmel et al. 2011). We recently reported that developmental exposures to prooxidants produce highly similar pancreatic structural anomalies. Embryonic exposure to \textit{tert}-butylhydroperoxide (tBOOH) resulted in hypomorphic islet area and elevated frequencies of islet variants in \textit{Tg(ins:GFP)} zebrafish eleutheroembryos and larvae (Sant et al. 2016b).

We also observed shortened ratios of exocrine pancreas length to total fish length, suggesting that embryonic MEHP exposure reduces exocrine mass, a phenotype that has been commonly associated with diabetes in human studies, known as dorsal pancreatic agenesis. Dorsal pancreatic agenesis is characterized by a shortened pancreatic tail, which leads to functional metabolic abnormalities such as hyperglycemia. Such functional abnormalities are primarily due to decreased endocrine tissue mass, as the majority of secondary islets are clustered within the distal body and exocrine tail, and thus a shortened length may impede their development (Bento et al. 2013). Reduced ratios of exocrine pancreas to total body length were similarly produced by developmental exposure to tBOOH in previous studies, indicating that oxidative stress may play a role in the development of this phenotype (Sant et al. 2016b).

Congruent with the hypomorphic pancreatic islets and shortened pancreas length observed in our microscopy experiments, expressions of the pancreas genes \textit{insa}, \textit{sst2}, and \textit{ptf1a} were significantly decreased at 96 hpf in MEHP-exposed fish compared to controls. Several trends emerged in gene expression following MEHP exposure. Expression of each endocrine hormone gene investigated was downregulated by MEHP exposure, consistent with reduced islet mass. However, while expression of \textit{ptf1a} and
crtb1 were also reduced, try and amy2a were upregulated, perhaps due to a compensatory or adaptive response to the toxicological insult.

We have demonstrated that exposure to MEHP during organogenesis clearly alters the structure of the pancreas, as well as expression of pancreas-related genes. However, the mechanism by which this toxicant is disrupting pancreatic development remains unknown. Previous work has shown that MEHP causes neurulation defects associated with altered GSH redox potential in mouse whole embryo cultures during organogenesis (Sant et al. 2016a). Based on this finding and other reports of MEHP-induced oxidative stress (e.g. (Meruvu, Zhang & Choudhury 2016, Tetz et al. 2013, Wang et al. 2012, Yang et al. 2012)), we hypothesized that oxidative stress is involved in the disruption of pancreas organogenesis by MEHP. We observed contrasting trends that indicate a more reduced potential for GSH, and a more oxidized potential for cysteine (Cys). Because GSH synthesis requires Cys, it is possible that the increased GSH and decreased Cys are due to shunting of Cys. However, total glutathione and total cysteine concentrations were both increased, suggesting that intracellular cysteine is mostly accumulating in its oxidized state. Likewise, ggtl (cysteine import), gclc (glutathione synthesis) and gsta1 expression were all unchanged due to treatment. Because gene expression and redox potentials were quantified 24 h following the most recently exposure timepoint, it is likely that embryos were able to mostly recover within this timeframe, as observed in mouse embryos (Sant et al. 2016a). Upon investigation of GSH-related gene expression, we found that MEHP altered expression of gsr and gstp1. We found that gsr was significantly upregulated, indicating that more cellular GSSG is present after MEHP exposure, which must be converted into GSH by gsr before it can be
used. Additionally, *gstp1* expression was significantly reduced by MEHP, which suggests that limited cellular detoxification is occurring due to lack of this enzyme. These results suggest that MEHP exposure is disrupting the cellular redox environment. Disruption of the sensitive redox balance can result in altered cell fate decisions, such as premature differentiation, which may have lasting consequences on both morphology and function (Rovira M. 2011). MEHP has been shown to produce the same structural changes in the endocrine and exocrine pancreas as classic prooxidants such as tBOOH, strengthening the evidence that MEHP is disrupting the cellular redox environment and resulting in altered pancreatic morphology (Sant et al. 2016b).

Numerous studies have associated chronic phthalate exposure with symptoms of metabolic dysfunction such as obesity, insulin resistance and hyperglycemia in humans. However, much remains to be learned about the biological mechanisms by which phthalate exposure may contribute to altered disease susceptibility (Sun et al. 2014). Here, we have shown that developmental exposures to the phthalate metabolite MEHP is capable of disrupting pancreatic organogenesis in zebrafish, reducing the size of the endocrine and exocrine pancreas, producing variant pancreatic morphologies, altering gene expression, and modifying the redox environment in the developing zebrafish embryo.
2.5. Supplemental Figures

Figure 7. Supplemental Figure 1. Embryonic MEHP exposure does not significantly alter expression of gcga, ghrl, or pdx1 at 96 hpf. AB zebrafish were exposed to MEHP from 3 to 96 hpf, and gene expression was assessed at 96 hpf. Data are presented as mean fold change normalized to the biweight mean center of bactin and b2m expression ± SEM (n = 6 pools of 10 fish; *p<0.05).
Figure 8. Supplemental Figure 2. Embryonic MEHP exposure does not significantly alter expression of *try*, *amy2a*, or *crtb1* at 96 hpf. AB zebrafish were exposed to MEHP from 3 to 96 hpf, and gene expression was assessed at 96 hpf. Data are presented as mean fold change normalized to the biweight mean center of bactin and b2m expression ± SEM (n = 6 pools of 10 fish; *p*<0.05).
Figure 9. Supplemental Figure 3. Embryonic MEHP exposure does not significantly alter the redox potential of GSH or Cys at 72 hpf. HPLC detection of soluble thiols and calculation of the Nernst equation revealed no significant changes µm GSH or Cys, total GSH or Cys, or redox potential of GSH or Cys at this timepoint (mean ± SEM; n = 7; *p<0.05).
Figure 10. Supplemental Figure 4. Embryonic MEHP exposure does not significantly alter expression of *ggt1*, *gclc*, or *gsta1* at 96 hpf. AB zebrafish were exposed to MEHP from 3 to 96 hpf, and gene expression was assessed at 96 hpf. Data are presented as mean fold change normalized to the biweight mean center of *bactin* and *b2m* expression ± SEM (n = 6; *p*<0.05).
CHAPTER 3

REDOX MODULATION OF ENDOCRINE PANCREAS GROWTH AND MORPHOLOGY DURING CRITICAL WINDOWS OF DEVELOPMENT IN THE ZEBRAFISH (Danio rerio)

This work is currently being prepared for publication with significant contributions from the following authors: Jiali Xu, Emily G. Severence, Karilyn E. Sant, Alicia R. Timme-Laragy.

3.1. Introduction

Early embryonic development is controlled by a uniquely sensitive network of signaling pathways which dictate the rapid growth and development necessary for an organism to survive. Relying on minute fluctuations in intracellular conditions, a developing embryo is highly susceptible to exogenous inputs which disrupt these sensitive pathways which direct cellular proliferation, differentiation, and apoptosis. The cellular redox environment has been recognized as a critical entity during early developmental stages. Changes in intracellular redox potential act as delicate “switches” through modification of cysteine sulfhydryl groups on regulatory proteins as the cellular redox potential shifts from reduced to oxidized conditions.

One of the most well-characterized redox-sensitive protein couples is glutathione (GSH) and its oxidized homodimer, glutathione disulfide (GSSG). Early embryonic development and organogenesis are characterized by periods of proliferation, differentiation, and apoptosis which are regulated in part by dynamic shifts in GSH redox potential occurring at specific stages during early developmental stages. Proliferation is favored under reducing cellular conditions (-250 to -225 mV), but gives way to differentiation as the cell becomes more oxidized (-220 to -200 mV). As oxidative conditions progress (>170 mV), apoptosis, or programmed cell death, is favored until the
cell becomes too oxidized to function normally (>145 mV) (Schafer & Buettner 2001, Hansen & Harris 2015, Jones 2002).

The condition in which normal redox signaling becomes disrupted by exogenous pro-oxidizing species is known as oxidative stress. Oxidative stress has been widely implicated in teratogenesis. One of the most well-known oxidative teratogens is thalidomide, an anti-morning sickness prophylactic prescribed to pregnant women in Europe during the mid 20th century, which resulted in widespread limb deformities in developmentally exposed populations (Therapontos et al. 2009). Additionally, embryonic exposure to the common environmental contaminant, methyl mercury, disrupts redox signaling and results in neurobehavioral deficiencies and cognitive impairment in offspring (Thompson et al. 2000). Oxidative stress has also been implicated in the etiology of many chronic diseases, such as cancer, cardiovascular disease, and diabetes (Jones 2006). Thus, maintaining a stable redox environment is critically necessary for successful regulation of these processes which govern organogenesis.

To maintain redox homeostasis, vertebrates have evolved a plethora of mechanisms to regulate the delicate balance between reactive oxygen species (ROS) and antioxidant enzymes which dictate cellular redox potential. One of these systems is the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, which responds to oxidative stress conditions by activating antioxidant response elements (AREs), which upregulate numerous genes, including those associated with GSH synthesis and recycling. Nrf2 is a cytosolic transcription factor; under normal cellular conditions it remains bound to Keap1, a redox-sensitive protein which keeps Nrf2 inactive through constitutive degradation. As the cell becomes increasingly oxidized, modification of Keap1 allows
Nrf2 to translocate to the nucleus and activate ARE transcription. Upregulation of antioxidant enzymes returns the cell to a more reduced, comfortable state, thus inactivating Nrf2 (Suzuki et al. 2015, Sant et al. 2017a).

Visualizing the effects of redox modulation during sensitive stages of embryonic development and organogenesis in vivo has been a challenge until recently. While cell culture and rodent models have provided an excellent framework for observing cellular changes produced by exogenous perturbation of the redox environment, little work has been done to characterize the structural consequences of oxidative stress in real time in vivo. The zebrafish model offers a unique solution to this issue. Zebrafish embryos are transparent, and develop externally to the mother, allowing for visualization of the entire developmental process from fertilization to organogenesis, as well as easy manipulation of the developmental media. Transgenic and mutant strains are widely available due to extensive characterization of the zebrafish genome, and allow for visualization of individual organ development in response to adverse redox conditions (Lele & Krone 1996, Yang et al. 2009, Kinkel & Prince 2009, Sant et al. 2017a).

We previously reported that the endocrine pancreas of the zebrafish is uniquely sensitive to oxidizing conditions produced by exogenous environmental toxicants during pancreatic organogenesis (Rousseau et al. 2015b, Timme-Laragy et al. 2015, Sant et al. 2016b). The endocrine pancreas, or the Islets of Langerhans, contains clusters of hormone-secreting cells found along the body of the larger exocrine pancreas. The function of the endocrine pancreas is to sense and respond to minute fluctuations in blood glucose levels, thus maintaining glucose homeostasis and regulating the energy balance of the organism. Thus, the high rate of perfusion in this organ is necessary for normal
function, but also results in a proportionately greater load of exogenous chemicals to be deposited in the pancreas. Many environmental toxicants have oxidizing physical properties, and the cells of the endocrine pancreas has an inherently low capacity for detoxification, due to a low basal expression of antioxidant genes compared to other cell types (Acharya et al. 2010). Consequently, it is postulated that the endocrine pancreas is distinctly sensitive to oxidative stress, especially during early developmental stages. However, the temporal windows of greatest susceptibility to the consequences of redox modulation during pancreatic organogenesis remain to be characterized.

In this study, we utilized an array of classical pro- and antioxidant compounds and Nrf2 activators, introduced to the developmental media at different stages of pancreatic organogenesis to elucidate the windows of greatest sensitivity in the zebrafish model. We observed the effects of redox modulation on the α-cells and β-cells, which secrete glucagon and insulin, respectively. These cell types compose the majority of the endocrine pancreas, and produce the master hormonal regulators of glucose homeostasis. The classical pro- and antioxidants used in this study were tert-butylhydroperoxide (tBOOH) and n-acetyl cysteine (NAC), respectively. We have previously shown that tBOOH exposure significantly reduces β-cell cluster area (Sant et al. 2016b). We compared the effects of these classical redox modulators to those produced by the established Nrf2 activators sulforaphane (SFN) and tert-butylhydroquinone (tBHQ). While SFN is considered an antioxidant, its function as such is mediated through upregulation of innate antioxidant defense mechanisms via activation of Nrf2 (Zhou et al. 2014). Thus, SFN acts as a weak pro-oxidant, while protecting against further oxidative damage by arming the cell with its own defense systems. tBHQ has been classified as a
potent Nrf2 activator through oxidative modification of Keap1 (Zagorski et al. 2013). By utilizing a number of compounds which act in different ways at different stages of development, this study aims to clarify the windows during which the endocrine pancreas is the most sensitive to disruption of the redox environment.

3.2. Materials and Methods

3.2.1. Chemicals

All chemicals used in this study were of the highest chemical grade available. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburg, PA, USA). Mono(2-ethylhexyl)phthalate (MEHP) was purchased from AccuStandard (New Haven, CT, USA). N-acetyl cysteine (NAC) and tert-butyl hydroperoxide (tBOOH) was purchased from Alpha Aesar (Ward Hill, MA, USA). Tert-butyl hydroquinone (tBHQ) was purchased from Acros Organics (NJ, USA). Sulforaphane (SFN) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared by diluting each chemical to the proper concentration in either 0.3x Danieau’s medium (Westerfield, 2007), or DMSO.

3.2.2. Animals

Adult zebrafish were maintained in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, with authorization from the University of Massachusetts Amherst Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3551-01). Fish were housed in a recirculating zebrafish system (Aquaneering, San Diego, CA, USA) at 28.5 °C on a daily 14 h light:10 hr dark cycle, and fed once daily with the recommended amount of Gemma Micro 300
(GM300) obtained from Skretting (Westbrook, ME, USA). Breeding fish populations were maintained in tanks containing approximately 20 individuals with a 1:2 ratio of males to females. Zebrafish embryos were collected daily from tanks approximately 0-1 hours post fertilization (hpf), washed thoroughly, and placed in 100 mm petri dishes of 0.3X Danieau’s solution.

*Tg(ins:GFP)* transgenic zebrafish on a wild type (AB) background were obtained from Dr. Philip dIorio at the University of Massachusetts Medical School (Worcester, MA, USA). *Tg(gcga:GFP)* transgenic zebrafish on an AB background were obtained from Dr. Jennifer Moss at Duke University (Durham, NC, USA). Used to visualize the development of the endocrine Islets of Langerhans, the *Tg(ins:GFP)* and *Tg(gcga:GFP)* strains co-express green fluorescent protein (GFP) with insulin and glucagon, allowing for in-vivo observation of the β-cells and α-cells, respectively (dIorio et al. 2002a, Pauls et al. 2007a). Wild type (nrf2a+/+) and nrf2a<sup>fh318/fh318</sup> (loss of function) zebrafish embryos on an AB strain background were used to investigate whether the detoxification of MEHP is governed by the Nrf2 pathway. Zebrafish heterozygous for the Nrf2a<sup>fh318</sup> mutation were generated by the TILLING mutagenesis project (R01HD076585), and obtained from Dr. Mark Hahn as embryos from the Moens Laboratory at the Fred Hutchinson Cancer Research Center (Seattle, WA, USA). Adults were bred in house to achieve homozygosity for wild type (nrf2a+/+) and mutant (nrf2a<sup>fh318/fh318</sup>) animals. Adult homozygous wild type and mutants were then crossed with *Tg(ins:GFP)* zebrafish to obtain wild type and mutant transgenics. Adult heterozygotes were then crossed and bred to homozygosity for the wild type and mutant genotype. All breeding adults were
genotyped as previously described to confirm homozygosity (Mukaigasa et al. 2012, Rousseau et al. 2015b).

3.2.3. Exposures

To investigate how the timing of pro- and antioxidant exposures impacts the development of the endocrine pancreas, zebrafish eleutheroembryos were exposed to NAC (100 µM), SFN (20 µM), tBOOH (77.5 µM), tBHQ (1 µM), water or DMSO (0.01%) control at either 24, 48, or 72 hpf. Following collection at approximately 3 hpf, all embryos were screened for fertilization. At 24 hpf, all eleutheroembryos were manually dechorionated using watchmaker’s forceps and screened under fluorescent light to confirm presence of the transgene. Eleutheroembryos were exposed to tBOOH for 10 minutes, tBHQ for 6 hours, and NAC, SFN, water, and DMSO for 24 hours before thorough washing in 0.3x Danieaus medium. Following exposure, fish were replaced in fresh 0.3x Danieaus medium and maintained for microscopy at 96 hpf.

3.2.4. Microscopy

Larvae were imaged at 96 hpf to observe the effects of exposure on the development of the primary islet, using an upright Olympus compound fluorescence microscope equipped with a Zeiss Axiocam 503 camera and Zen analysis software (Zeiss, USA). Fish were briefly anesthetized in 0.3x Danieaus containing MS-222 and staged laterally in 3% methylcellulose to optimize visualization of the endocrine islet. Images were captured using monochrome and GFP fluorescence filters at 2X, 5X, and 10X magnification to assess gross morphology and islet structure. Immediately following imaging, fish were washed thoroughly in fresh 0.3x Danieau’s medium and allowed to fully recover. Images were blinded, then analyzed using Zen (Zeiss) analysis software.
Islet area was determined by tracing the perimeter of the cell cluster on the 10X GFP images, and the area was calculated by the software. Islet morphology was also assessed to quantify the frequency of islet variants such as fragmented, stunted, hollow islets, or ectopic endocrine cells (Sant et al. 2016b).

3.2.5. Statistics

Statistical analysis was performed using Microsoft Excel and R statistical software, version 3.4.1. To assess statistical significance, Analysis of Variance (ANOVA) tests, followed by a Fishers Least Significant Differences (LSD) Post-Hoc Test, were performed in R. All experiments were repeated for 5-7 biological replicates, with 5-10 individuals per trial (total n=30). Data are presented as the mean plus or minus the standard error of the mean (SEM).

3.3. Results

3.3.1 Nrf2a\(^{(fh318/fh318)}\) Mutant Endocrine Islet Morphometrics

We have previously shown that embryonic exposure to MEHP impairs the development of the pancreas. To determine whether the observed disruptions to pancreatic organogenesis may be due in part to MEHP-induced oxidative stress, we used a Nrf2a\(^{(fh318/fh318)}\) mutant zebrafish line, characterized by a loss-of-function Nrf2 response to oxidative conditions due to a point mutation in the DNA binding domain. These mutant fish have been previously characterized to be more sensitive to oxidative stress, and more susceptible to adverse effects of toxicant exposures that involve oxidative stress (Rousseau et al. 2015a, Mukaigasa et al. 2012). No significant changes in gross
morphology (length, yolk sac area, pericardial area) were observed in wild type or Nrf2a^{fh318/fh318} mutants. Islet morphometrics were additionally reexamined as in the previous chapter using wild type and mutant (Nrf2a^{fh318/fh318} Tg(ins:GFP) transgenic lines. We observed reductions in β-cell cluster area across genotype, consistent with findings from the previous islet morphometrics experiment, and an increase in the variant β-cell morphologies greater than that found in wild type MEHP-exposed embryos was observed in the MEHP-exposed Nrf2a mutant embryos at 72 hpf. The distribution of variant morphologies also differed between the Nrf2a mutant and wildtype embryos exposed to MEHP. For example, at 48 hpf, MEHP exposure resulted in mainly stunted and hollow islet morphologies in the wildtype embryos, but mainly stunted islets in the Nrf2a mutants (Fig. 1).

3.3.2. 24 hpf Exposure

Modulation of the redox environment by exogenous prooxidant exposure at 24 hpf resulted in significant decreases in β-cell cluster area and significant increases in the frequency of islet morphological variants at 96 hpf. tBOOH exposure at 24 hpf produced

**Figure 11.** Embryonic exposure to MEHP significantly reduced β-cell area and increased the frequency of pancreatic islet variants in wt and Nrf2a^{fh318/fh318} homozygous mutant fish (mean ± SEM; n = 30; means sharing a letter in the group label are not significantly different at the 5% level).
the most significant decrease in β-cell cluster area (17.73%, p<0.05), while tBHQ exposure produced the most significant increase in the frequency of morphological variants at 96 hpf, compared to the average of the controls (26.67%, p<0.05). tBOOH exposure at 24 hpf resulted in a slight increase in variant frequency (8.72%) at 96 hpf. While the frequency of ectopic β-cells remained consistent with that of the controls, tBOOH also produced an increased frequency of fragmented and stunted islets, although this increase was not of statistical significance (p>0.05). tBHQ exposure at 24 hpf

![Figure 12](image)

**Figure 12.** Exposure to pro-oxidants tBOOH and tBHQ at 24 hpf decreased β-cell cluster area and increased the frequency of pancreatic islet variants at 96 hpf. Exposure at 24 hpf did not significantly alter α-cell cluster area (mean ± SEM; n=30; means sharing a letter are not significantly different at the 5% significance level). Methods schematic is modified from Tiso et al. 2009.

reduced β-cell cluster area by approximately 12.62% compared to the controls at 96 hpf (p<0.05). While the decrease in islet area was not as dramatic as that of the tBOOH exposure group, tBHQ additionally resulted in a significant increase in the frequency of fragmented, stunted, and hollow islets.
Antioxidant exposures produced no significant changes in β-cell cluster area at 24 hpf, however NAC exposure increased the frequency of morphological islet variants, specifically ectopic β-cells and hollow islets, by approximately 10% compared to the average of the controls. SFN exposure at 24 hpf produced no changes in either β-cell cluster area or variant frequency.

The water and DMSO vehicle control treatments did not produce any significant differences in islet area or variant frequency at this exposure timepoint (p>0.05). The average frequency of morphological islet variants in the controls was approximately 6.67%, and the only islet variant observed were ectopic β-cells. From these results, it is apparent that prooxidant exposure at 24 hpf affects endocrine pancreas development (Fig. 2).

3.3.3. 48 hpf Exposure

At 48 hpf, modulation of the redox environment by the introduction of pro- and antioxidants to the developmental media resulted in significant alterations in both β-cell cluster area and the frequency of morphological islet variants.

At 48 hpf, redox modulation by prooxidant exposure significantly reduced β-cell cluster area compared to the controls at 96 hpf (p<0.05). Exposure to tBOOH resulted in a 22.83% decrease in β-cell cluster area, while tBHQ exposure reduced the area by 26.45% compared to the controls (p<0.05). Congruent with the trend of decreased islet area, prooxidant exposure at this timepoint produced an elevated frequency of stunted islet morphologies, and the total frequency of islet variants was significantly increased by both tBOOH and tBHQ exposures. Compared to a background frequency of approximately 3% islet variants, all ectopic β-cells, tBOOH and tBHQ exposures
produced variance frequencies of 30.3% and 36.67%, respectively. While stunted islets were the most common islet variant observed at this exposure timepoint, both prooxidant exposures produced hollow islets as well, and tBHQ exposure additionally produced

![Figure 13.](image)

Figure 13. Exposure to antioxidants and prooxidants at 48 hpf increased and decreased β-cell cluster area, respectively. Prooxidants increased the frequency of pancreatic islet variants, and significantly reduced β-cell cluster area at 96 hpf (mean ± SEM; n=30; means sharing a letter are not significantly different from one another at the 5% significance level). Methods schematic is modified from Tiso et al. 2009.

Antioxidant exposure at 48 hpf significantly increased β-cell cluster area compared to controls at 96 hpf (p<0.05). NAC exposure at 48 hpf resulted in a 20.68% increase in β-cell cluster area (p<0.05), and slightly increased the frequency of ectopic β-cells (3.64%, p>0.05) compared to the controls at 96 hpf. Similar to NAC exposure at 48 hpf, SFN increased β-cell cluster area by 25.7% compared to the controls (p<0.05).

Contrary to the effects observed in the 24 hpf exposure group, SFN exposure at 48 hpf
produced a greater frequency of morphological islet variants than NAC at this exposure timepoint, with a 10.3% increase in variant frequency compared to the controls.

No significant changes were observed between the islet areas and variant frequencies between the DMSO and water controls at this exposure timepoint (p>0.05). These results indicate that redox modulation by the addition of both pro- and antioxidants at this sensitive stage of development is highly capable of modifying the development of the endocrine pancreas (Fig. 3).

3.3.4. 72 hpf Exposure

Redox modulation at 72 hpf by the pro- and antioxidants tBHQ and SFN significantly altered β-cell cluster area, and all pro- and antioxidant exposures produced a significantly elevated frequency of islet morphological variants compared to the controls at 96 hpf.

Prooxidant exposure at 72 hpf resulted in decreased β-cell cluster area and highly elevated frequencies of morphological islet variants. Similar to the previous exposure timepoints, tBOOH exposure reduced islet area by approximately 18% (p<0.05), and produced a wide range of morphological islet variants, including ectopic β-cells, fragmented, hollow, and stunted islets, increasing the frequency of total islet variants by 24.5% compared to the controls (p<0.05). tBHQ exposure also significantly reduced β-cell cluster area by 21.86% (p<0.05), and this effect was congruent with an 18.8% increase in the frequency of stunted islets compared to the controls (p<0.05).

Exposure to the antioxidants NAC and SFN at 72 hpf produced highly different results in β-cell cluster area. Contrary to the previous exposure timepoints, NAC exposure at 72 hpf reduced β-cell cluster area compared to the controls, but this effect
was not statistically significant at a less than 10% decrease in area (p>0.05). NAC exposure additionally produced a slight increase in the frequency of stunted islets compared to controls (6.66%, p>0.05). Compared to NAC, SFN exposure at 72 hpf resulted in the opposite effect on β-cell cluster area, but had a similar effect on islet variant frequency. At 72 hpf, SFN exposure increased islet area by 8.52%, but simultaneously increased the frequency of stunted islets by 10% (p>0.05). While not statistically significant, these trends suggest that SFN is capable of producing highly variable effects on endocrine islet size at this developmental stage.

No significant differences in islet area or variant frequency were observed between the water and DMSO controls at this exposure timepoint (p>0.05). These results

**Figure 14.** Exposure to SFN and tBHQ at 72 hpf increased and decreased β-cell cluster area, respectively. Prooxidants increased the frequency of pancreatic islet variants at 96 hpf. Exposure at 72 hpf did not significantly alter β-cell cluster area at 96 hpf (mean ± SEM (n=30; means sharing a letter are not significantly different from one another at the 5% level). Methods schematic is modified from Tiso et al. 2009.
indicate that redox modulation at this timepoint may produce different structural effects on the endocrine pancreas than at other timepoints (Fig. 4).

3.4. Discussion

The early stages of development inherently depend on a dynamic network of sensitive signaling pathways which dictate the periods of rapid cellular proliferation and tissue organization. Disruption of this network by exogenous agents during these critical windows of development can result in adverse birth outcomes, and may predispose them to various health conditions later in life. The cellular redox environment has been characterized as one of these important developmental networks, the disruption of which has been associated with adult onset diseases such as cardiovascular disease, obesity, and diabetes (Hansen & Harris 2013, Hansen & Harris 2015).

Until recently, much of the research at the intersection of redox and developmental biology has largely focused on overt teratogenic outcomes. Pioneer redox biologists showed that oxidative stress induced by exposure to pharmaceutical drugs and alcohol consumption during pregnancy impaired normal development and resulted in both morphological and functional deficits at birth (Hansen et al. 2004, Devi et al. 1993). While the establishment of drugs and alcohol as teratogens led to many important public health initiatives to protect developing infants, it is rapidly becoming apparent that perturbation of the redox environment during these early stages can also result in far more subtle changes to organ structure and function that do not become apparent until disease onset during adulthood.
We have previously shown that exposure to environmental contaminants such as polychlorinated biphenyls (PCBs), perfluoroalkyl substances (PFAS), and phthalates during early developmental stages can disrupt pancreatic organogenesis in the zebrafish, resulting in reduced mass of both endocrine and exocrine tissues, and altered expression of pancreatic hormone genes. We have additionally shown that these compounds disrupt the redox environment and alter GSH signaling, therefore we suspect that oxidative stress is likely a mechanism of toxicity for these chemicals (Timme-Laragy et al. 2015, Sant et al. 2017b, Sant et al. 2016b). In the previous chapter, we showed that the phthalate metabolite MEHP is capable of reducing both α- and β-cell cluster mass, shortening exocrine tissue length, and disrupting GSH-related gene expression. The present study aims to elucidate both the temporal windows of greatest susceptibility during which the zebrafish is most sensitive to fluctuations in the redox environment.

Previous studies have identified an association between MEHP-induced oxidative stress and Nrf2 induction. In rat INS-1 cells, treatment with DEHP resulted in significantly increased ROS production, dysregulation of the Nrf2-mediated antioxidant response, and inhibition of glucose-stimulated insulin secretion (Sun et al. 2015). The results of our microscopy assay using wild type and Nrf2α<sup>fh318/fh318</sup> mutants showed a similar decrease in β-cell cluster area that was largely due to exposure rather than genotype, with the exception being a transient increase in aberrant β-cell cluster morphology in the Nrf2a mutant fish exposed to MEHP assessed at the 72 hpf time point. Similar trends in the wild type and mutant strains indicate that embryonic MEHP exposure may affect organogenesis of the endocrine pancreas through a mechanism independent of Nrf2a, or perhaps the other Nrf-related proteins are able to compensate for
deficient Nrf2a, as has been shown for the regulation of the glutathione system during zebrafish development (Sant et al. 2017a). To answer some of these questions, we deliberately modified the redox environment at specific stages of zebrafish pancreas development to identify a sensitive timepoints at which exposure to pro- or antioxidant compounds may impair endocrine pancreas development. By utilizing both classical redox modulators and Nrf2 activators, we were able to discern both stage- and chemical-specific effects of exposure on the endocrine pancreas.

We observed significant reductions in β-cell cluster area in response to prooxidant exposure. Exposure to tBHQ consistently produced the largest decrease in β-cell cluster area at all exposure timepoints, most significantly when exposed at 48 hpf. tBHQ exposure additionally produced the greatest number of “stunted,” or hypomorphic, islet phenotypes, which may account for the significantly diminished mean β-cell cluster area. tBOOH also significantly reduced β-cell cluster and increased the frequency of morphological islet variants. Our findings are supported by previous reports that tBHQ produce an overt oxidative stress response (OSR) in zebrafish, during which fundamental antioxidant defense genes, including Nrf2, are rapidly upregulated to mitigate oxidative damage (Hahn et al. 2014, Kobayashi et al. 2009, Timme-Laragy et al. 2009). Hahn et al. reported an elicit tBHQ-induced OSR, demonstrated by increased levels of GSH-related gene expression over the same developmental time scale. Expression of gstp1 and gcld, both genes which regulate the synthesis and recycling of GSH, was significantly increased at 48 hpf in response to both tBOOH and tBHQ, supporting the notion that 48 hpf is a sensitive developmental window for both whole-embryo and pancreas-specific outcomes of redox modulation by prooxidant exposure (Hahn et al. 2014, Timme-Laragy
et al. 2009). Interestingly, α-cell cluster area did not appear to be affected by prooxidant exposure to the degree seen in the β-cells, nor were any morphological variants detected. This suggests that β-cells are uniquely sensitive to redox modulation by exogenous prooxidant exposure, a finding also supported by in vitro studies which demonstrate a low basal antioxidant capacity, and are thus acutely sensitive to oxidative stress (Tiedge et al. 1997). While the results of this study indicate that prooxidant exposure at these stages does not affect the α-cells, results from the previous chapter showed that MEHP exposure did, in fact, produce hypomorphic α-cell cluster area, which suggests that MEHP may impact pancreatic organogenesis in more ways than just oxidative stress induction. More research is needed to address how MEHP exposure impacts α-cell development.

We also observed both chemical- and stage-specific pancreatic responses to antioxidant exposure. Neither NAC nor SFN affected α- or β-cell cluster area when zebrafish embryos were exposed at 24 hpf, although a slight increase in the frequency of islet variants was observed in response to NAC exposure compared to the controls. At 48 hpf however, both NAC and SFN significantly increased β-cell area, further implicating this timepoint as a sensitive window for redox modulation in the reducing direction as well. At 72 hpf, NAC had no effect on β-cell cluster area, while slightly increasing α-cell cluster area, while the opposite effect was observed in response to SFN exposure. While both considered antioxidant compounds, these chemicals work in different ways to replenish GSH within the cell. NAC directly increases available cysteine levels to promote GSH synthesis, while SFN indirectly restores GSH levels by inducing cellular antioxidant response genes such as Nrf2 (Zhou et al. 2014, Timme-Laragy et al. 2009).
Previous work has shown that NAC exposure is capable of rescuing prooxidant-induced effects on GSH levels, but only at certain developmental stages (Timme-Laragy et al. 2009). We expect that control of the β-cell antioxidant response to redox modulation changes over the developmental time course to incorporate the Nrf2 antioxidant defense system, although further studies are necessary to make this assumption. However, this would lend to the explanation of why NAC and SFN appear to act inversely at the 48 and 72 hpf exposure timepoints.

Our discovery of 48 hpf as a sensitive developmental window for pancreas-specific effects of redox modulation is supported by literature which shows that fusion of the posterodorsal and anteroventral buds of the pancreas occur around this timepoint (Tiso et al. 2009a). Simultaneously, a distinct decrease in whole-embryo GSH redox potential can be observed at this timepoint, which may increase the organisms susceptibility to oxidative damage at 48 hpf (Timme-Laragy et al. 2013). Disruption of this fusion by altered redox signaling would likely produce altered β-cell cluster mass, and may weaken normal organ function later in the life course. We have shown that exposures to compounds which modulate the redox environment during pancreatic development have differential capacity to alter the structure of the endocrine pancreas in different ways when exposures occur at different stages of development. We have identified 48 hpf as a sensitive window of development, during which altered redox signaling significantly changes the structure of the β-cell cluster, while the α-cell cluster remained intact. In conclusion, these findings indicate that the β-cells are uniquely sensitive to oxidative stress during the early stages of development, specifically at 48 hpf.
CHAPTER 4

CONCLUSIONS

Results from this research project indicate that exposure to environmental chemicals which modify the redox environment during sensitive developmental windows disrupts pancreatic organogenesis. While there is not sufficient evidence to conclude that MEHP alters pancreatic architecture entirely via oxidative stress, it is apparent that an oxidative stress response, induced at critical developmental stages, can produce similar structural abnormalities in the endocrine pancreas.

Exposure to prooxidizing agents, including MEHP, significantly reduced β-cell cluster area, and increased the frequency of morphological islet variants. MEHP additionally reduced the area of the α-cell cluster, although this effect was not produced by the other prooxidants, suggesting that MEHP may additionally affect the pancreas by redox-independent mechanisms. The effect of MEHP exposure on the endocrine pancreas was also seen in the altered the expression of several endocrine hormone genes in the glucoregulatory axis, which suggests that endocrine function could be affected as well. Further studies are necessary to determine whether these changes in gene expression actually translate to altered pancreatic function.

Exposure to redox modulators at 48 hpf resulted in the most significant fluctuations in β-cell cluster area, suggesting that this may be a uniquely sensitive window for pancreas development. No significant differences in redox potentials of GSH or Cys due to MEHP exposure were detected, however MEHP did appear to alter the expression of genes involved in the recycling and synthesis of GSH, most notably *gstp1* and *gsr*, indicating that redox signaling may indeed be altered by MEHP exposure. It is
still unclear whether Nrf2 is involved in detoxification of MEHP, as our Nrf2a knock-down fish did not appear to be overtly more sensitive to exposure. Future studies should focus on alternative signaling pathways which may be involved in the pancreatic effects of MEHP exposure seen in this project. MEHP additionally reduced the ratio of exocrine pancreas to total fish length, and altered expression of exocrine hormone genes. Future studies plan to evaluate the effects of redox modulation on the exocrine pancreas to determine if oxidative stress may be involved in exocrine pancreas insufficiency induced by MEHP and other contaminants. It would also be of interest to determine the sensitive windows of development of the exocrine pancreas, and whether these are similar or different to those of the endocrine pancreas.

While it appears as though these compounds significantly alter pancreatic architecture, further research is necessary to determine whether these structural abnormalities persist past these early developmental stages and result in impaired pancreas function later in the lifecourse. Future studies intend to address whether developmentally-induced structural anomalies persist, and if these morphological variants can ultimately correct themselves once the exposure is removed. Due to their rapid development, quick reproductive turnover and ability to regenerate tissues, the zebrafish model will continue to serve as an ideal model for studying the later-life effects of developmental exposures to environmental chemicals. Very little is still known about the environmental contributions to metabolic disease, and whether developmental toxicant exposures play a role in predisposing individuals to adult-onset illness. While future research is critically necessary to explore these possibilities, results from these studies aim to contribute to our understanding of how a common environmental toxicant, MEHP,
affects pancreatic organogenesis, and the role of the redox environment in this relationship.


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