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Effects of Butylparaben Exposure on Pancreatic Development in Zebrafish (Danio rerio) Embryos

Sarah E. Brown
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Effects of Butylparaben Exposure on Pancreatic Development in Zebrafish (*Danio rerio*) Embryos

A Thesis Presented

by

SARAH E. BROWN

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

MASTER OF SCIENCE

September 2016

Environmental Health Sciences
Effects of Butylparaben Exposure on Pancreatic Development in Zebrafish (Danio rerio) Embryos

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I would like to thank my thesis advisor, Dr. Alicia Timme-Laragy, for this incredible opportunity. Working with Dr. Timme-Laragy has been life-changing, and through her guidance and with her support, I have learned so much and have accomplished milestones that I before only dreamed of accomplishing. I want to thank Dr. Timme-Laragy for always being there for me. The challenges that she has put in front of me have given me the confidence that I can overcome any challenges I may encounter in the future. I feel extraordinarily lucky to have had the opportunity to work for her and with her.

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ABSTRACT

EFFECTS OF BUTYLPARABEN EXPOSURE ON PANCREATIC DEVELOPMENT IN ZEBRAFISH \textit{(Danio rerio)} EMBRYOS

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SARAH E. BROWN, B.S., SAINT MICHAEL’S COLLEGE

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Butylparaben (Butyl p-hydroxybenzoic acid) is a widely used cosmetic and pharmaceutical preservative that has been recently shown to induce oxidative stress and have endocrine disrupting effects in rodents, and promote adipocyte conversion of human adipose cells. Embryonic development is extremely sensitive to oxidative stress due to changes in cell growth, development and differentiation that occur during this life stage. Fluctuations in redox potentials play critical roles in normal embryonic development by guiding these cell signaling, cell-fate decisions and apoptosis. The most prevalent endogenous antioxidant that defends against oxidative stress is glutathione (GSH), which scavenges reactive oxygen species. The low antioxidant capacity of pancreatic beta cells suggests that they are sensitive target tissues of oxidative stress; this has yet to be investigated during embryonic development. Here, we aim to 1) determine whether embryonic exposure to butylparaben prompts structural and functional changes in the developing endocrine pancreas and 2) determine whether oxidative stress may be involved. Transgenic \textit{insulin-GFP} zebrafish embryos were treated daily with 250, 500, 1,000 and 3,000 nM butylparaben starting at 3 hours post fertilization (hpf). Pancreatic islet and whole embryo morphological development were examined daily until 7 days post fertilization (dpf). Redox potentials were measured at 24 and 28 hpf using HPLC.
Area of the pancreatic islet increased over time with increasing butylparaben exposure in a dose-dependent manner by as much as a 55% increase in islet area at 3 dpf when compared to controls. Butylparaben concentrations of 500 and 1,000 nM increased GSH by 10 and 40%, respectively, and decreased oxidized glutathione disulfide by 37 and 59%. GSH redox potentials were only significant in embryos collected at 28 hpf and became more reduced with 500 and 1,000 nM butylparaben exposure, decreasing redox potentials by 7 and 18 mV, respectively. Cysteine redox potentials also became more reduced, decreasing by 17 and 28 mV. Our data show that butylparaben-induced redox potential disruptions that may be responsible for the effects on pancreatic islet structure and function, but further studies are needed to determine how and if that directly affects pancreas development.
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CHAPTER 1

INTRODUCTION

1.1. Diabetes

Over the past few decades, the incidence of diabetes has been rapidly increasing worldwide. According to the Centers for Disease Control, in the United States alone, the prevalence of diagnosed diabetes has increased 176% from 1980 to 2011. Type 2 diabetes (T2D) is currently the seventh leading cause of death in the United States (Pitkaniemi, et al 2004). Diabetes has often been characterized as a disease that affects the populations of developed countries, resulting from a sedentary lifestyle. However, according to the International Diabetes Federation, today four out of five people with diabetes now live in developing countries. According to the World Health Organization, people in developing countries are also exposed to greater amounts of environmental pollutants. This increased prevalence of T2D diabetes in genetically stable populations indicates the presence of a strong environmental component that is influencing this escalation (Pitkaniemi et al 2004). T2D develops from impaired insulin signaling that leads to the pancreas’s inability to maintain normal glucose homeostasis, and compensate for the body’s resistance to insulin. This compensation often fails due to mitochondrial dysfunction and endoplasmic reticular stress, leading to beta cell apoptosis (Makaji et al 2011). Developmental exposures to environmental factors may affect the development of the pancreas, predisposing individuals to T2D later in life.
1.2. The Pancreas

The pancreas is an endoderm-derived organ that consists of both exocrine and endocrine tissues. The exocrine acinar tissues are mainly responsible for producing digestive enzymes, whereas the endocrine islets of Langerhans produce hormones that maintain glucose homeostasis. The pancreatic islets are comprised of a central core of beta cells that produce insulin, surrounded by alpha, delta and epsilon cells that produce the hormones glucagon, somatostatin and ghrelin respectively (Kinkel et al, 2009). The hormones of the endocrine pancreas work together to maintain glucose homeostasis: insulin inhibits the secretion of glucagon from alpha cells, while glucagon activates the secretion of insulin and somatostatin from beta and delta cells, whereas somatostatin and ghrelin inhibit insulin secretion (Figure 1) (Jiang et al, 2011).

Figure 1. The endocrine pancreatic hormones insulin, somatostatin, glucagon and ghrelin are responsible for the regulation of glucose homeostasis. Insulin (mainly responsible for glucose homeostasis) inhibits glucagon secretion from alpha cells. Glucagon activates the secretion of insulin and somatostatin, whereas somatostatin and ghrelin inhibit insulin secretion. (Jiang et al, 2011)
The zebrafish pancreas is structurally similar to the mammalian pancreas, and shares the same cellular makeup (Kinkel et al., 2009). During development, the pancreas arises from the endoderm germ layer (from which the liver, gallbladder and intestine are also derived) and differentiates into the exocrine and endocrine tissues (Warga et al., 1999). At the 24 somite stage of the developing zebrafish, the islet precursor cells of the pancreas arise from endodermal progenitor cells and bud off dorsally from the gut, giving rise to endocrine tissues that express insulin, along with other pancreatic hormones (Tiso et al., 2009). Between 24 and 48 hours post fertilization (hpf), the rotating gut dislocates this dorsal bud on the right side of the gut, forming an islet that is organized similarly to the mammalian islet by 48 hpf (Tiso et al., 2009 and Biemar et al., 2001). The mature zebrafish endocrine pancreas forms around 7 days post fertilization (dpf) and consists of an organized mass of beta cells, surrounded mainly by alpha and delta cells that make up the primary islet (Figure 2), and smaller secondary islets that are nested in the exocrine tissues (Tiso et al., 2009).

Mammalian in utero systems do not allow for the direct observation of in vivo organ, especially pancreatic, development. Organogenesis cannot be directly visualized within the mammalian uterus, and tissue sampling is often invasive. In contrast, zebrafish embryos are see-through, and do not develop in utero, allowing for the direct observation of organ and whole-embryo development in vivo. As a result, the zebrafish model is becoming increasingly used for developmental research, due to this species' rapid developmental growth, the numerous existing transgenic lines, and transparent embryos that allow for real time imaging of morphologic development (Lieschke et al., 2007). Because of these characteristics, the zebrafish has become a well-established model used
to study human development and disease, including pancreatic development and
developmental glutathione redox dynamics (Tiso et al, 2009 and Timme-Laragy et al,
2013).

The Transgenic (ins-GFP) zebrafish line was created so that insulin expression is
represented by Green Fluorescent Protein (GFP) (diIorio et al, 2002). This line was first
generated by injecting approximately 5 nL containing 20 ng/L cesium-purified plasmid
DNA and 50 ng/mL Tc3A transposase mRNA into zebrafish embryos at the one to two
cell stage, creating single-copy insertions (diIorio et al, 2002). As a result, fluorescence
occurs whenever preproinsulin is transcribed, creating GFP as a marker in pancreatic beta
cells. Because beta cells produce insulin, during embryonic development, this is an ideal
strain that allows for the visualization and imaging of the endocrine pancreas in vivo in
real-time.

Figure 2. Location, organization and composition of the adult zebrafish pancreatic islet.
(Kinkel et al, 2009)

1.3. Oxidative Stress

One factor implicated in T2D is oxidative stress. As defined by Jones et al, 2006,
oxidative stress is the “disruption of redox signaling and control.” Intrauterine oxidative
stress is known to be associated with an increased risk of developing T2D later in life
(Simmons et al, 2006). Due to their low expression of antioxidant enzymes, such as
superoxide dismutase, catalase, glutathione and glutathione peroxidase, pancreatic beta
cells are especially sensitive to oxidative stress (Lenzen et al, 1996). The most prevalent
endogenous antioxidant that defends against oxidative stress is glutathione (GSH), a
tripeptide of cysteine, glutamate and glycine. GSH is synthesized via two ATP-dependent
steps: 1) the enzyme glutamate-cysteine ligase (Gcl) combines glutamate and cysteine in
the rate-limiting step; 2) a glycine is added to the glutamate-cysteine molecule in a
reaction catalyzed by glutathione synthase (Gss) (Figure 3). GSH defends against
oxidative stress by scavenging reactive oxygen species (ROS) which oxidize the cysteine
moiety, and by acting as a co-factor for the antioxidant enzymes glutathione-S-transferase
(Gst) and glutathione peroxidase (Gpx) (Timme-Laragy et al, 2013). When GSH is
oxidized, it dimerizes and becomes glutathione disulfide (GSSG), which can be recycled
back into reduced glutathione (GSH) in a NADPH-dependent reaction, catalyzed by the
enzyme glutathione disulfide reductase (Gsr) (Figure 3) (Timme-Laragy et al, 2013).

Measured concentrations of GSH and GSSG can be applied to the Nernst equation
to calculate redox potentials (Eₗ) (Jones et al, 2006). Fluctuations in redox potentials play
an important role in normal embryonic development by guiding cell signaling, cell-fate
decisions and apoptosis (Hansen et al, 2013). More positive redox potentials indicate a
more oxidized redox state and are associated with cell differentiation and apoptosis,
whereas more negative redox potentials suggest a more reduced redox state and are
associated with cell proliferation (Schafer et al, 2001). Embryonic exposure to
environmental anthropogenic chemicals, such as parabens, may generate ROS that
disturb redox signaling and affect intracellular redox potentials, altering cell fate
decisions, and potentially leading to disrupted pancreatic islet structure and function.
1.4. Butylparaben

Parabens (para-hydroxybenzoic acids) are a family of alkyl esters that are currently the most widely used preservatives in cosmetic and pharmaceutical products due to their extensive range of antimicrobial activity. In fact, parabens are the most common cosmetic ingredient after water (Cashman et al., 2005). Butylparaben is one of the most commonly used parabens in commercial products after methyl- and propylparaben. However, only a few studies have been conducted that investigate an association between butylparaben and oxidative stress. Butylparaben has been found to induce oxidative stress in the liver tissues of mice by significantly decreasing GSH (43.87%) along with the enzymatic activities of GSH peroxidases and GSH-S-transferases (Shah et al., 2012). A study that measured GSH redox dynamics in the brain tissues of developmentally exposed mouse offspring also found GSH and the GSH/GSSG percent ratio to decrease and GSSG and percent GSSG to increase, suggesting the occurrence of oxidative stress (Hegazy et al., 2015). Urinary butylparaben levels have
been shown to be associated with biomarkers of oxidative stress in humans (Kang et al., 2013). Watkins et al (2015) also found urinary butylparaben concentrations to be significantly associated with 27 percent higher isoprostane (a urinary marker of oxidative stress) and weakly associated with OHdG (a marker of oxidative stress to DNA).

Often combined to enhance antimicrobial effects, parabens differ due to the specific R-group at the para position on the molecule’s benzene ring (Figure 4). The most commonly used parabens are methyl-, ethyl, propyl-, benzyl-, and butyl-paraben. The different alkyl groups give parabens a spectrum of antimicrobial effects with antimicrobial activity and lipophilicity increasing with the length of the alkyl group (Cashman et al, 2005). Parabens have been found to act as preservatives by increasing the impermeability of bacterial cell membranes to proteins and other ions, and by penetrating the phospholipid bilayer of bacterial cell membranes, displacing the phospholipid molecules (Eklund et al, 1980). Parabens also obstruct amino acid uptake in bacterial membrane vesicles, inhibiting bacterial growth, and have been found to interfere with both RNA and DNA synthesis in bacteria (Eklund et al, 1980 and Nes et al, 1983).
Humans are most commonly exposed to parabens dermally. However, after all routes of exposure, parabens are metabolized by carboxylesterases in the skin, liver, linings of the intestine, and subcutaneous fat into metabolites: p-carboxyphenyl glucuronide, p-hydroxybenzoyl glucuronide, and p-carboxyphenyl sulfate (Cashman et al, 2005 and Soni et al, 2005). These carboxylesterase enzymes are both expressed and active in zebrafish embryos as early as the 6 somite stage (Kuster et al, 2005). Conventionally, it has been assumed that parabens are quickly absorbed and metabolized, which originally contributed to the belief that parabens have low human toxicity. However, parabens have been found to escape metabolism in the skin and intestine and have been found in human urine, blood serum, and breast milk samples. Ye et al (2006) discovered free, un-metabolized butylparaben in 69% of urine samples from subjects with no known occupational exposures. Free butylparaben (an average of 135 µg/L) was also found in the serum of young male volunteers who were exposed to cosmetics containing
butylparaben for one week (Janjua et al, 2007). Jimenez-Diaz et al (2011) also detected an average of 0.4 ng/g\(^{-1}\) free butylparaben in human placental tissues.

A 1995 survey conducted on 215 cosmetic products found parabens in 99% of leave-on cosmetics and 77% of rinse-off products (Rastogi et al, 1995). As of 2005, the United States population is exposed to an average of 76 mg of parabens per day, with butylparaben being the third most abundant exposure (Cashman et al, 2005). Early estimates of human exposures to butylparaben in the United States due to long-term cosmetic use range from 0.26 to 14.6 mg/day (Masten, et al 2005). In 2014, estimates of the daily usage of cosmetic products that contain parabens were 17,760 mg for adults and 378 mg for infants (Anderson, 2008). According to the United States Food and Drug Administration’s Center for Food Safety and Applied Nutrition, the United States population consumes an average rate of 37 ng butylparaben per day. Parabens have also been detected in adult urine samples as well as in the amniotic fluid of pregnant women (0.3 µg/L) (Philippat et al, 2013), suggesting the potential for direct human fetal exposure to parabens.

Parabens have also been shown to have endocrine disrupting effects. Butylparaben has been found to interfere with the male and female reproductive system by acting as a weak estrogenic chemical, and by hindering spermatogenesis. Butylparaben was found to compete with [3H] estradiol for binding to estrogen receptors in female rats, and revealed estrogenic responses in sexually mature rainbow trout (Routledge et al, 1998, Pedersen et al, 2000). In both male rats and mice, butylparaben exposure lowered serum testosterone in a dose-dependent manner, while decreasing sperm count and daily sperm production (Oshi et al, 2001, and Oshi et al, 2002).
Butylparaben has also been found to increase the release of the adipocyte hormones leptin, resistin, and adiponectin through increased peroxisome proliferator-activated receptor (PPARγ) activation (Taxvig et al, 2012). PPARγ is mainly expressed in adipose tissues where it influences the storage of fatty acids and induces adipocyte differentiation (Tontonoz et al, 1995). Because PPARγ plays a role in the regulation of adipocyte differentiation, Taxvig et al show that, through PPARγ activation, butylparaben stimulates lipid accumulation in differentiating adipocytes, which results in an increase in the release of adipocyte related hormones. Hu et al (2013) also found butylparaben to enhance adipocyte differentiation, increasing glucocorticoid activity in addition to activating PPARγ. The potency of these effects was amplified with increasing paraben alkyl chain length, with butylparaben displaying the largest magnitude of effect.

Obesity is known to be associated with an increased risk in developing T2D (Kahn et al, 2006). The hormones leptin, adiponectin and resistin are all associated with increased insulin resistance and increased risk for developing diabetes (Taxvig et al, 2012). Increased levels of circulating leptin has been associated with obesity, however low fetal leptin levels may increase the risk of obesity and insulin resistance later in life (Djiane et al, 2008). Adiponectin shows an inverse association with insulin resistance (Taxvig et al, 2012). Resistin has been shown to regulate insulin sensitivity and metabolism in different tissues (Steppan et al, 2001). Due to its interactions with estrogen receptors, interfaces with hormones that are associated with insulin resistance, and increased adipocyte differentiation, butylparaben may disrupt endocrine system function.
To date, no studies have been conducted that examine the effects of butylparaben in the zebrafish model (*Danio rerio*); however studies have been conducted using propylparaben. Mikula *et al.* (2008) found that juvenile zebrafish exposed to 500 mg/kg⁻¹ propylparaben, administered in feed three times daily, influenced sex differentiation by significantly skewing the sex ratio towards the female sex, suggesting propylparaben’s ability to interfere with gonad development. Because sex differentiation in zebrafish is influenced by hormones, this skewed sex ratio is most likely due to propylparaben’s interference with the hypothalamo-pituitary-gonadal axis, suggesting endocrine disrupting effects of parabens in vertebrates.

It is unknown whether butylparaben affects the developing pancreas. A preliminary study in 1956 found butylparaben esters recovered in the pancreas of dogs that were intra-venously administered 100 mg/kg butylparaben per day (Jones *et al.*, 1956). However, no further studies have been conducted to investigate whether butylparaben affects the developing pancreas. Because pancreatic beta cells have low antioxidant levels and gene expression, they are more likely susceptible to oxidative damage than the other cell types in the exocrine pancreas. This thesis tests the hypothesis that daily butylparaben exposure will alter the development of the endocrine pancreas with the possible involvement of oxidative stress.

**1.5. Thesis Overview**

This study aims to determine whether butylparaben may prompt structural and functional changes in the beta cells of the endocrine pancreas, and to investigate whether butylparaben induces oxidative stress in the developing zebrafish. Chapter 2 examines how embryonic butylparaben exposure affects endocrine pancreatic development and
whole embryo morphological development in transgenic (ins-GFP) zebrafish. Embryos were imaged at 3, 4, 5 and 7 days post fertilization; islet areas were measured and islet deformities were quantified to determine whether butylparaben affects islet development by altering islet area and morphology. Yolk sac area was also measured throughout development to indicate metabolism and to determine the rate at which exposed embryos utilize nutrients. Other morphological deformities were also quantified to determine whether pancreatic islets are specifically sensitive to butylparaben. Gene expression of the endocrine pancreatic hormone index was also quantified.

Chapter 3 identifies the impact of butylparaben on glutathione redox dynamics in embryonic zebrafish. GSH and GSSG were quantified to determine redox potentials in transgenic (ins-GFP) zebrafish embryos. Embryos endured exposures to 500 nM and 1,000 nM butylparaben and were collected for redox analysis at 24 and 28 hpf. Redox potentials were determined using HPLC detection of soluble thiols. Expression of the genes involved in glutathione synthesis, and utilization, including glutamate cystine ligase subunits (gclc and gclm), glutathione synthetase (gss), glutathione disulfide reductase (gsr) and glutathione S-transferase (gst) were measured at 3 days post fertilization via qPCR, using β-actin as a housekeeping gene. These studies aim to determine: 1) whether embryonic exposure to butylparaben prompts structural and functional changes in the developing endocrine pancreas and 2) determine whether butylparaben induces oxidative stress that may contribute to islet morphology.

Regarding the gene and protein name formatting, the approved format for designing genes and proteins (see the ZFIN Zebrafish Nomenclature Web site https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines) was used. Human genes and proteins are labeled using all capitals (INSA and INSA respectively), and rodent genes and proteins are labeled Insa and INSA respectively. Zebrafish genes and proteins are characterized as insa and Insa respectively.
CHAPTER 2

BURYLPARABEN ALTERS THE DEVELOPING PANCREAS

2.1. Introduction

Over the past few decades, the incidence of both Type 1 and Type 2 diabetes has greatly increased in genetically stable populations, suggesting influential environmental factors are contributing to this escalation. Early life exposures to environmental chemicals may impact the development of the endocrine pancreas, and may contribute to this escalated prevalence (Pitkaniemi et al, 2004). The pancreas is highly vascularized, and is therefore likely exposed to environmental chemicals that are in the bloodstream. Two main tissues make up the pancreas: the exocrine acinar tissues that produce digestive enzymes, and endocrine islets of Langerhans that are composed of different cells that produce hormones involved in the regulation of glucose homeostasis (Jiang et al, 2011). The alpha cells of the endocrine islets produce glucagon that signals the degradation of molecules such as glycogen for the production of free glucose. Epsilon and delta cells produce ghrelin and somatostatin respectively, which negatively regulate insulin and glucagon secretion. The beta cells of the endocrine pancreas are the only cells that produce insulin, which promotes the absorption of glucose from the bloodstream (Jiang et al, 2011). The transcription factor Pdx1 transcribes for both insulin and glucagon.

The zebrafish pancreas is structurally similar to the mammalian pancreas and shares this same cellular make-up (Kinkel et al, 2009). The zebrafish model is commonly used in developmental toxicological research, especially due this model’s plethora of
different transgenic lines. Here, using the transgenic ins-GFP line, the developing pancreas can be observed in vivo in real time.

Butylparaben has been recovered in pancreatic tissues, and has been found in human amniotic fluid, suggesting the possibility of direct human fetal and pancreas exposure (Jones et al, 1956 and Philippat et al, 2013). To date, there are no studies that observe whether butylparaben affects the developing pancreas. This study aims to identify whether daily exposure to different environmentally relevant concentrations of butylparaben affect the development of the endocrine pancreas in the zebrafish model. Embryos were exposed daily to different environmentally relevant concentrations of butylparaben and were imaged for pancreas and morphological deformities at time points that are critical to pancreatic and embryonic development. Islet areas were also measured and the incidences of islet deformities were calculated.

The developing islet is easily disrupted by any morphological deformities that may be a consequence of butylparaben exposure. Therefore, embryonic gross development was analyzed by measuring nose-to-tail embryonic length and yolk sac area to indicate the embryos’ utilization of nutrients. The incidence and severity of morphological deformities were also accounted for in a deformity index, and the occurrence of morphological deformities were compared to the incidence of islet deformities. Gene expression of the hormones involved in the regulation of insulin homeostasis (glucagon, somatostatin, insulin and ghrelin) were measured along with the transcription factor Pdx1. These experiments aim to determine whether butylparaben prompts structural changes in the developing zebrafish pancreas.
2.2 Materials and Methods

2.2.1 Chemicals

Butylparaben (Acros Organics, Thermo Fisher Scientific, Pittsburg, PA) was acquired as a generous gift from Dr. Ling Zhao (Department of Nutrition, University of Tennessee Knoxville, Knoxville, TN) dissolved in 0.01% v/v Dimethyl Sulfoxide (DMSO) (Fischer Scientific, Pittsburg, PA). Solutions were stored at -20 ºC and vortexed before each use.

2.2.2 Zebrafish Husbandry

*Tg (ins-GFP)* embryos were obtained from Dr. Phillip diIorio at the University of Massachusetts Medical School Zebrafish Facility (Worcester, MA). This strain was chosen due to its specific expression of green fluorescent protein in the beta cells of the endocrine pancreas. Heterozygous *nrf2a* \(^{fh318}\) embryos, that were crossed to obtain the wildtype *nrf2a* \(^{+/+}\) embryos used in these experiments, were generated by the Tilling Mutagenesis Project, obtained from the Moens Laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA) and acquired as a generous gift from Dr. Mark Hann of the Woods Hole Oceanographic Institute (Woods Hole, MA).

All zebrafish used in these experiments were maintained under 14 hours of light and 10 hours of darkness at 28.5°C in an Aquaneering system, and adults were fed a diet of Gemma Micro granule fish food (Skretting, Westbrook, ME) daily. Embryos were collected from group matings of three 6 liter tanks of approximately 20 fish per tank with a 1:2 male to female ratio. Embryos were maintained at low densities (approximately 30 embryos) in 10 mL 0.3x Danieau’s solution (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO\(_4\), 1.8 mM Ca(NO\(_3\))\(_2\), 1.5 mM HEPES, pH 7.6) (Westfield *et al*, 2007). Embryo water was
changed daily. All procedures are approved by the University of Massachusetts Amherst IACUC committee (Animal Welfare Assurance Number A3551-01).

2.2.3. Survivorship

Wildtype zebrafish of the AB line, obtained from Boston Children’s Hospital (Boston, MA), were used to observe survivorship. Embryos were exposed to butylparaben concentrations of 1,000, 3,000 and 5,000 nM butylparaben, suspended in 0.01% (v/v) Dimethyl Sulfoxide (DMSO). This solvent was selected due to its lack of toxicity in animal models. Butylparaben concentrations were chosen after a preliminary dose range-finding experiment saw complete mortality following exposure to 10,000 nM butylparaben.

Chemical exposures began at 3 hours post fertilization (hpf) and were replicated daily until 72 hpf. This exposure regime was used for two key reasons: 1) the literature suggests that parabens are rapidly metabolized, within 24 hours of exposure in rabbit models (Soni et al., 2005), and 2) it is assumed that the majority of human paraben exposure is due to cosmetic applications, so re-dosing daily will most accurately mimic human exposure patterns.

Embryos were collected and screened approximately one hour after fertilization and were divided into four groups, each group containing 30 embryos. Each group of 30 embryos was placed in a glass Petri dish containing 20 mL of 0.3x Danieau’s solution and 0.01% (v/v) DMSO, 1,000, 3,000 or 5,000 nM butylparaben. Embryos were then individually placed into 96-well plates, with 200 µL of their respective dosed water, and imaged using Evos FL auto inverted fluorescent microscope (Life Technologies, Pittsburgh, PA) to check for fertilization. Exposures and whole-embryo imaging was
repeated at 24, 48 and 72 hpf with one trial of approximately 27-30 embryos per treatment. Embryos were maintained in 96-well plates throughout the experiment. Observations of mortality, heartbeat, yolk sac coagulation, hatching status, and pericardial and yolk sac edema were recorded at each time point.

2.2.4. No Observed Effect Concentration (NOEC)

In order to determine the no observed effect concentration (NOEC) of butylparaben exposure to the pancreatic islet, Tg (ins-GFP) embryos were exposed daily to concentrations of 62.5, 125 and 250 nM butylparaben. Embryos were imaged and analyzed at 4 days post fertilization. Exposures and imaging were carried out as described above. Images were taken at 40x magnification to screen embryos for developmental malformations, and at 100x and 200x magnification under trans and GFP overlaid filters to image pancreatic islets. Islet areas were measured blindly using EVOS software and islet deformities were quantified. This procedure was repeated twice with 10 embryos per treatment.

2.2.5. Chemical Exposures

In order to expose embryos to a wide range of butylparaben concentrations in both imaging and gene expression experiments, embryos were exposed to 0.01% (v/v) DMSO or 250, 500, 1,000, or 3,000 nM butylparaben in 20 µL of 0.3x Danieu’s solution. These concentrations were chosen after preliminary data from the survivorship study were analyzed due to their low production of morphological abnormalities and low mortality rates. Exposures began at 3 hpf and were renewed daily with water changes (0.3x Danieu’s solution) until 7 days post fertilization (dpf). Embryos in all experiments were manually dechorionated at 1 dpf using watchmaker’s forceps.
2.2.6. Microscopy

Islets, islet morphology and embryonic developmental morphology were imaged in Tg (ins-GFP) embryos at 3, 4, 5 and 7 dpf. Images were obtained with an EVOS Auto-FL Inverted microscope (Life Technologies, Pittsburgh, PA). At each imaging time point, embryos were anesthetized with MS222 (tricaine) in 0.3x Danieau’s solution and then placed into individual drops of 3% methylcellulose in a right lateral orientation. Images were taken at 20x and 40x magnification under a trans filter, and at 100x and 200x magnification under trans and GFP filters using Evos FL auto inverted fluorescent microscope (Life Technologies, Pittsburgh, PA). After imaging, all embryos were washed in 20 mL of 0.3x Danieau’s, re-dosed with their respective treatment and stored at 28.5°C.

2.2.7. Imaging Analysis

All islet images used in islet area analysis were taken at 200x magnification, and islet area was measured blindly using EVOS software. Islets that were not in focus or that were occluded by pigmentation were excluded (approximately 1-4 embryos per concentration per trial). Three categories of islet deformities were observed: islet fragmentation (where the beta cells of the islet are more dispersed), ectopic beta cells (where a beta cell emerges away from the primary islet), and “fuzzy” islets (whose fuzzy appearance is not an artifact of the imaging process). To quantify whether butylparaben affected embryonic gross development, yolk sac area (a surrogate measure of the embryo’s ability to uptake nutrients) and whole embryo nose-to-tail lengths (to measure embryo growth) were measured using EVOS software. Because the swim bladder is
derived from the same progenitor endoderm cells as pancreatic beta cells, delayed swim bladder inflation was also evaluated (Kimmel and Meyer, 2010).

A total deformity index was created to quantify the five developmental deformities observed with butylparaben exposure. These deformities include: pericardial edema (fluid retention in the membrane surrounding the heart), decreased yolk sac utilization (increased yolk sac contents), intestinal effusion (fluid retention in the intestinal lumen), craniofacial malformations and spinal malformations. Each deformity was scored on a scale from 0-3, with 0 as normal, 1 for a mild deformity, 2 for a moderate deformity and 3 for a severe deformity. A deformity index was then calculated as the sum of scores for each individual embryo in each dose group at each time point divided by the maximum score possible, and multiplied by 100. This approach is in agreement with other deformity assessments in fish embryo models (Harbeitner et al, 2013, Wassenberg et al, 2004, and Whitehead et al, 2010).

2.2.8. RNA Extraction and Reverse Transcription

Wildtype nrf2a+/+ embryos (5 biological replicates of approximately 10-12 embryos per treatment) were exposed daily to DMSO, 250, 500, 1,000 or 3,000 nM butylparaben and collected for RNA isolation at 3 days post fertilization. Samples were stored at -80°C in 100 µL RNA Later. Prior to RNA extraction, all materials and surfaces were sprayed with RNase Away® (Molecular Bio Products). RNA extractions were carried out according to the GeneJET RNA Purification Kit Total RNA Purification Protocol (Thermo Fisher Scientific Inc., Waltham, MA). Samples were sonicated for approximately one second at 15 % amplification with a Branson Digital Sonifier® (Danbury, CT) that was sterilized with 75% ethanol between each sonication. RNA
concentration and quality was analyzed using a BioDrop µLITE (BioDrop, Cambridge, UK). Reverse transcription to cDNA was carried out according to the manufacturer’s protocol using an iScript cDNA synthesis kit for reverse transcription PCR (Bio-Rad Laboratories, Hercules, CA) with a final concentration of RNA of 500 ng/15 µL RNA. Synthesis was then carried out in an Eppendorf Mastercycler® nexus gradient thermal cycler (Eppendorf, Hauppauge, NY) according to the reaction protocol provided by iScript.

2.2.9. Quantitative real-time PCR

To gain insight about the function of beta cells, the transcription of genes in the pancreatic endocrine hormone axis was measured. Gene expression of the pancreatic hormones preproinsulin a (insa), glucagon a (gcga), ghrelin (ghrl), and somatostatin 2 (sst2) and the transcription factor pancreatic duodenal homeobox 1 (pdx1) were quantified with β-actin utilized as a housekeeping gene. β-actin is a known housekeeping gene used with the zebrafish model, and its expression has not been shown to change due to chemical exposures (McCurley and Callard, 2008).

All primers were first tested for specificity and efficiency. Primers were designed using Primer-BLAST (NCBI) and were optimized for melting temperature and for dimerization, to avoid both homodimer and heterodimer dimerization. cDNA was diluted to a working concentration of 0.125 ng/µL, as previously performed by Rousseau et al (2015). A 20 µL PCR reaction was carried out that contained 0.5 µL of each foreword and reverse primer, 10 µL of 2x iQ™ SYBR® Green Supermix (Bio-Rad), 5 µL of nuclease-free water, and 4 µL of diluted cDNA. Quantitative real-time PCR (qPCR) was performed in a Bio-Rad CFX Connect™ real-time system under the conditions of 2
minutes at 95°C followed by 10 seconds at 95°C, and then 25 seconds at 60-68°C depending on the melting temperature of each primer. A dissociative curve was created for each primer run. The primer sequences (5’-3’ orientation) used for the pancreas-related genes gcga, insa, pdx1 and housekeeping gene β-actin are listed in Table 1. The primers for sst2, and ghrl were purchased from Bio-Rad (PrimePCR™). Data obtained from qPCR were analyzed using the Bio-Rad CFX Connect Manager™ software, version 3.0 (Bio-Rad). The ddCT method was used to calculate fold change.

Table 1. Primer sequences of pancreas-related genes gcga, insa, pdx1 and housekeeping gene β-actin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 5’-3’</th>
</tr>
</thead>
</table>
| gcga   | Foreword: GGACAAACCAAACCAGATGGTT  
|        | Reverse: CCTGACGCTCTCGAGTTTCAT  |
| insa   | Foreword: GCCCAACAGGCTTCTTCTCAAC  
|        | Reverse: GCAATTTAGGAGGAAGGAAACCC  |
| pdx1   | Foreword: CATCTCTACAGTGCTCGGG  
|        | Reverse: ACCATATAAGGGCCTGTCAC  |
| β-actin| Foreword: CAACAGAGAGAAGATGACACAGATCA  
|        | Reverse: GTCACACCATTACTACAGGAGTCCATCAC  |

2.2.10. Statistical Analysis

Data was analyzed using Microsoft Excel and Stata statistical software, version 14.1. All statistical tests were performed by Stata. Two-factorial ANOVAS followed by a Tukey’s post hoc test or a Wald’s post hoc test were used to test for effects among exposed groups and controls. Logistic regression analysis was performed to determine significance for swim bladder data and a Mann-Whitney U test was used to analyze the deformity index. Probit analysis was performed to analyze survivorship. A McNemar’s
test for significance was used to determine independence between islet deformities and morphological deformities. Data are presented as the mean and the standard error of the mean (SEM).

2.3. Results

2.3.1. Survivorship

In order to assess embryonic survivorship in response to daily butylparaben exposure, survivorship analysis was performed daily until 3 days post fertilization. The control (DMSO) and 1,000 nM groups retained 96% and 92% survivorship throughout all time points respectively. Survivorship in the 3,000 nM group decreased from 88% to 85% survivorship at 3 dpf. Survivorship in the 5,000 nM group decreased from 89% to 85% over the three day exposure (Figure 5).

![Survivorship graph](image)

**Figure 5. Survivorship of wildtype embryos after exposure to 3 different concentrations of butylparaben.** Thirty embryos per treatment were exposed to 0.01% (v/v) DMSO, 1,000, 3,000 or 5,000 nM butylparaben. Survivorship was observed daily until 3 days post fertilization.
2.3.2. Butylparaben Disturbs Pancreatic Islet Development

To determine the low-dose toxicity of butylparaben on islet development, the NOEC was calculated for islet area and defects. Islet area increased in a dose-dependent manner (Figure 6). However, this trend was only significant at the highest concentration (250 nM) (p< 0.05). Here, the lowest observed effect level concerning islet area was 250 nM, and the no observed effect level was 125 nM. Butylparaben also induced islet deformities at 250 nM, with 70% of islets displaying a malformation compared to 20% of embryos exposed to 125 nM, and 0% of embryos exposed to 62.5 nM and 22 % of the controls (Table 2).

![Figure 6. Islet areas are sensitive to butylparaben down to concentrations of 250 nM at 4 dpf. Transgenic (ins-GFP) embryos were exposed daily to 62.5, 125 and 250 nM butylparaben (n= 15-20 embryos per treatment). Imaging was performed at 4 dpf under a trans and GFP filter using EVOS imaging software. Islet areas were measured using EVOS software editing tools. p<0.05.](chart.png)
Table 2. Incidences of total and specific islet deformities in embryos exposed daily to DMSO, 62.5, 125, or 250 nM butylparaben at 4 dpf.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>62.5 nM</th>
<th>125 nM</th>
<th>250 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Defects</strong></td>
<td>3/14 (21%)</td>
<td>1/19 (5%)</td>
<td>4/16 (25%)</td>
<td>9/17 (53%)</td>
</tr>
<tr>
<td>Fragmented</td>
<td>2/14</td>
<td>1/19</td>
<td>1/16</td>
<td>1/17</td>
</tr>
<tr>
<td>Ectopic</td>
<td>1/14</td>
<td>0/19</td>
<td>3/16</td>
<td>5/17</td>
</tr>
<tr>
<td>Fuzzy</td>
<td>0/14</td>
<td>0/19</td>
<td>0/16</td>
<td>3/17</td>
</tr>
</tbody>
</table>

In order to quantify beta cell development, the area of islet fluorescence was measured at the time points of 3, 4, 5 and 7 days post fertilization. Butylparaben exposure was found to significantly increase islet area by as much as 55%. Islet area increased significantly for all concentrations of butylparaben when compared to controls at 3 dpf (p< 0.05) (Figure 7). Embryos exposed to 250 nM butylparaben had islet areas that were significantly larger than controls at all time points (3, 4, 5 and 7 dpf). At 4 dpf, islet area was significantly greater in embryos exposed to 250 and 500 nM butylparaben, and at 5 dpf only embryos exposed to the lowest concentration of 250 nM saw a significant increase in islet area. At 7 dpf, islet area was significantly greater in embryos exposed to the lowest (250 nM) and highest (3,000 nM) concentrations of butylparaben (p<0.05) (Figure 7).

Islet area was also seen to increase over time until 5 dpf and then decrease at 7 dpf. However, exposure to 3,000 nM butylparaben decreased islet area at all time points when compared to areas at 3 dpf (Figure 8).
Figure 7. Daily exposure to butylparaben increases islet area over 7 days. Transgenic ins-GFP zebrafish were exposed daily to 250, 500, 1,000, and 3,000 nM butylparaben and imaged under a GFP filter using EVOS Auto-FL Inverted microscope software at 3, 4, 5 and 7 days post fertilization. Islet areas were measured using the EVOS software editing tools (n= 15-25 embryos). A one-factor ANOVA followed by a Tukey’s post hoc test was utilized to test for effects among exposed groups and controls. Means sharing a letter in the group label are not significantly different at the 5% level.
Figure 8. Islet area decreases over time with butylparaben treatment. Transgenic ins-GFP zebrafish were exposed daily to 250 nM, 500 nM, 1,000, and 3,000 nM butylparaben and imaged under a GFP filter using EVOS Auto-FL Inverted microscope software at 3, 4, 5 and 7 days post fertilization. Islet areas were measured using the EVOS software editing tools (n= 15-25 embryos). A two-factorial ANOVA followed by a Wald post hoc test was used to test for effects among exposed groups and controls (p<0.05).

Exposure to butylparaben also resulted in irregular development of the pancreatic islet that was observed at 3-7 dpf. The three most prevalent deformities observed were fragmentation of the beta cells, where the beta cells of the islet appear more dispersed (Figure 9.B.), ectopic beta cells, in which a beta cell emerges away from the primary islet (Figure 9.C.) and “fuzzy” islets, where the fuzzy appearance is not an artifact of the imaging process (Figure 9.D.). The incidence of these deformities changed with butylparaben exposure. Fragmented islets were most prevalent at the 250 nM concentration (44%), whereas ectopic beta cells were most prevalent at 500 nM (16%), and “Fuzzy Islets” were predominant at 3,000 nM (54%) (Table 3). Between 3 and 7 dpf, 90% of control embryos displayed normally developed islets compared to 53% of the 250
nM group, 75% of the 500 nM group, 71% of the 1,000 nM group, and 67% of embryos exposed to 3,000 nM groups. Three day post fertilization embryos that were exposed to 250 nM and 3,000 nM butylparaben had the greatest incidence of total islet defects (56% and 58% respectively) (Table 3). Islet fragmentation decreased over time, whereas the prevalence of “Fuzzy Islets” appeared to decrease over time at the higher concentrations of 1,000 nM and 3,000 nM, but increased at the lower concentrations of 500 nM and 250 nM butylparaben (Table 3). There are no obvious trends relating to the incidence of ectopic beta cells. It is important to note that these malformations may contribute to increased islet size.
<table>
<thead>
<tr>
<th>Total Defects</th>
<th>3 dpf</th>
<th>4 dpf</th>
<th>5 dpf</th>
<th>7 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>2/23 (9%)</td>
<td>3/23 (13%)</td>
<td>3/23 (13%)</td>
<td>1/21 (4%)</td>
</tr>
<tr>
<td>Fragmented</td>
<td>1/23</td>
<td>2/23</td>
<td>1/23</td>
<td>0/23</td>
</tr>
<tr>
<td>Ectopic</td>
<td>0/23</td>
<td>1/23</td>
<td>2/23</td>
<td>0/21</td>
</tr>
<tr>
<td>Fuzzy</td>
<td>1/23</td>
<td>0/23</td>
<td>0/23</td>
<td>1/23</td>
</tr>
<tr>
<td><strong>250 nM</strong></td>
<td>9/16 (56%)</td>
<td>8/16 (50%)</td>
<td>7/16 (44%)</td>
<td>6/16 (38%)</td>
</tr>
<tr>
<td>Fragmented</td>
<td>7/16</td>
<td>7/16</td>
<td>5/16</td>
<td>4/16</td>
</tr>
<tr>
<td>Ectopic</td>
<td>1/16</td>
<td>1/16</td>
<td>2/16</td>
<td>0/16</td>
</tr>
<tr>
<td>Fuzzy</td>
<td>1/16</td>
<td>0/16</td>
<td>0/16</td>
<td>2/16</td>
</tr>
<tr>
<td><strong>500 nM</strong></td>
<td>8/25 (32%)</td>
<td>4/24 (16%)</td>
<td>5/24 (21%)</td>
<td>7/24 (29%)</td>
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<tr>
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<td>3/25</td>
<td>2/24</td>
<td>0/24</td>
<td>2/24</td>
</tr>
<tr>
<td>Ectopic</td>
<td>4/25</td>
<td>2/24</td>
<td>2/24</td>
<td>1/24</td>
</tr>
<tr>
<td>Fuzzy</td>
<td>1/25</td>
<td>0/24</td>
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<td>4/24</td>
</tr>
<tr>
<td><strong>1,000 nM</strong></td>
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<td>9/25 (36%)</td>
<td>4/25 (16%)</td>
<td>6/25 (24%)</td>
</tr>
<tr>
<td>Fragmented</td>
<td>4/25</td>
<td>5/25</td>
<td>0/25</td>
<td>2/25</td>
</tr>
<tr>
<td><strong>3,000 nM</strong></td>
<td>15/26 (58%)</td>
<td>11/26 (42%)</td>
<td>6/26 (23%)</td>
<td>2/26 (8%)</td>
</tr>
<tr>
<td>Fragmented</td>
<td>1/26</td>
<td>0/25</td>
<td>1/25</td>
<td>1/26</td>
</tr>
<tr>
<td>Ectopic</td>
<td>0/26</td>
<td>1/25</td>
<td>2/25</td>
<td>0/26</td>
</tr>
<tr>
<td>Fuzzy</td>
<td>14/26</td>
<td>10/25</td>
<td>2/25</td>
<td>1/26</td>
</tr>
</tbody>
</table>

Table 3. Incidences of specific and total islet deformities: Fragmented, Ectopic and Fuzzy.
2.3.3. Developmental Toxicity of Butylparaben

To determine the effects of butylparaben on embryonic development, embryos were analyzed for developmental deformities. *Tg (ins-GFP)* embryos were exposed daily to concentrations of 250, 500, 1,000 and 3,000 nM butylparaben, and were imaged at 3, 4, 5 and 7 dpf. Five developmental deformities were used in a deformity index: pericardial edema, underutilized yolk sac, intestinal effusion, craniofacial malformations and spinal malformations (Figure 10.B). Butylparaben increased total deformities in a dose-dependent manner. Embryos exposed to the lowest concentration, 250 nM, did not show more deformities when compared to the controls at any time point. The highest concentration, 3,000 nM, significantly increased total deformities at all time points (p<0.0001). Embryos exposed to 500 nM butylparaben only showed more deformities at 3 and 7 days post fertilization, and those exposed to 1,000 nM saw significantly more deformities at 3, 4, and 7 dpf (Figure 10.A). At 3 dpf, butylparaben exposures greater than 250 nM significantly increased total deformities when compared to the controls.
(Figure 10.A.). It is important to note that some embryos displayed multiple deformities. However most embryos were not deformed at all, and the majority of embryos that were deformed did not have any islet defects.

A.
Figure 10. Deformity index for total morphological deformities and examples of morphologic deformities. A. Deformity index for total morphologic deformities. (Max Deformity Score = 100) Total developmental deformities including pericardial edema, underutilized yolk sac, intestinal effusion, craniofacial malformations and spinal
malformations. Tg(ins-GFP) embryos were exposed to 250, 500, 1,000 and 3,000 nM butylparaben daily (5-10 embryos per treatment for 4 trials) and imaged using EVOS Imaging software at 40x magnification. *=p<0.05, **=p<0.0001. B. Examples of morphologic deformities. Specific developmental deformities including pericardial edema, underutilized yolk sac, intestinal effusion, craniofacial malformations, and spinal malformations increase in severity from 1-3 at 3-7 dpf. Images were taken at 40x magnification in Tg(ins-GFP) embryos exposed daily to DMSO, 250, 500, 1,000, and 3,000 nM butylparaben.

To observe the effects of butylparaben on gross embryonic development, yolk sac utilization and embryonic growth were quantified. Embryonic yolk sac areas were measured at 3, 4 and 5 dpf to measure nutrient uptake. We found no significant relationship between butylparaben concentration and yolk sac area. Although a trend in smaller yolk sac area was seen throughout all time points at the 250 nM exposure, this trend was not significant (Figure 11.A.). Embryonic growth was quantified by measuring nose-to-tail linear length. Similarly to yolk sac area, embryonic body length was not significantly affected by butylparaben at any concentration from 3-7 dpf (Figure 11. B.).
In addition to embryonic growth, swimbladder inflation in response to chemical exposures is an endpoint that is often observed in the zebrafish model. Because the teleost swim bladder is derived from the same progenitor endoderm layer as the pancreas, we examined the effect of butylparaben on swim bladder inflation at 4, 5, and 7 days post fertilization. Throughout all time points, there was a trend in increased swim bladder inflation at 250 nM when compared to controls, though this trend was only significant at 4 dpf. Swim bladder inflation significantly decreased at the highest concentration (3,000 nM) by 40%, 62%, 50% when compared to the controls at 4, 5 and 7 dpf respectively (p < 0.05) (Figure 12). The concentrations of 500 and 1,000 nM butylparaben did not affect swim bladder inflation when compared to the controls.

![Figure 12. Percent swimbladder inflation at 4, 5 and 7 dpf. Tg (ins-GFP) embryos were treated daily with 250, 500, 1,000 and 3,000 nM butylparaben (n=30 embryos per treatment). Inflated swimbladders were quantified for each treatment. Logistic regression was used to determine differences among control and exposed groups (p<0.05).](image)

To further investigate whether these developmental morphologies may be contributing to the occurrence of islet deformities, the relationship between the specific
morphologic deformities (swimbladder inflation, intestinal effusion, spinal malformations, decreased yolk sac utilization, pericardial edema, and craniofacial malformations) and islet architecture was observed using a McNemar’s test for independence. P-values produced by this test that are greater than 0.05 indicate the relationship between the specific morphologic deformity and the occurrence of islet deformities occur independently. P-values that are less than 0.05 indicate a lack of independence. Here, the relationship between specific morphologic deformities and the occurrence of pancreas deformities was measured in control groups and embryos that were exposed to 250 and 3,000 nM butylparaben at 4 dpf. No clear relationship was observed between the occurrence of developmental deformities and islet architecture (Figure 13). However, it is interesting to note that these relationships change based on exposure (Figure 13).

<table>
<thead>
<tr>
<th>Deformity</th>
<th>Controls</th>
<th>250 nM</th>
<th>3,000 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swimbladder Inflation</td>
<td>0.005</td>
<td>0.054</td>
<td>0.001</td>
</tr>
<tr>
<td>Intestinal Effusion</td>
<td>0.083</td>
<td>0.256</td>
<td>0.124</td>
</tr>
<tr>
<td>Spinal Malformations</td>
<td>0.157</td>
<td>0.005</td>
<td>0.003</td>
</tr>
<tr>
<td>Decreased Yolk Utilization</td>
<td>0.561</td>
<td>0.008</td>
<td>0.739</td>
</tr>
<tr>
<td>Pericardial Edema</td>
<td>0.650</td>
<td>0.005</td>
<td>0.739</td>
</tr>
<tr>
<td>Craniofacial-malformations</td>
<td>1.000</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

**Figure 13. The relationship between morphologic deformities and islet architecture.**
The relationship between specific morphologic deformities and the occurrence of islet deformities in control groups and embryos that were exposed to 250 and 3,000 nM butylparaben at 4 dpf (n= 30 embryos per treatment). A McNemar’s test for independence was performed to investigate whether these relationships are independent.

**2.3.4. Expression of Pancreas-related Genes**

To assess the effects of butylparaben on beta cell function, the gene expression of the pancreatic endocrine hormone axis was measured. Gene expression of the pancreatic hormone index: *preproinsulin a (insa)*, *glucagon a (gcga)*, *ghrelin (ghrl)*, and
somatostatin 2 (sst2) was measured using a real-time quantitative polymerase chain reaction (qPCR). The transcription factor pancreatic duodenal homeobox 1 (pdx1) was also measured, and β-actin was utilized as a housekeeping gene. There was no significant relationship between butylparaben exposure and the gene expression of the pancreatic hormone index. However, the expression of pdx1 was significantly downregulated at the highest concentration (3,000 nM) (Figure 14).

![Gene Expression (Fold Change) vs Butylparaben (nM)](chart)

**Figure 14. Pdx1 is downregulated at the 3,000 nM butylparaben exposure.** Wildtype embryos were exposed daily to DMSO, 250, 500 or 3,000 nM butylparaben, and were collected at 78 hpf. Expression of pdx1 was measured and the ddCt method was used to calculate fold change, followed by pairwise T-tests to calculate significance (p< 0.05). N= 5 biological replicates per group with each replicate containing pools of 10-13 embryos.

**2.4. Discussion**

The endocrine pancreas is highly vascularized and is therefore likely exposed to chemicals, such as parabens, that may be in the blood stream. There is great potential for human exposure to butylparaben, and it has been recovered in pancreatic tissues and in human amniotic fluid at concentrations of 0.3 µg/L. In this study, I hypothesized that butylparaben would affect the development of the pancreatic islet. To test this hypothesis,
I analyzed whether butylparaben alters the development of the endocrine pancreas while observing butylparaben’s effects on gross development in zebrafish embryos.

The data from this study indicate butylparaben affects islet development by increasing islet area and increasing the incidence of islet deformities. The cause of this increasing islet area with butylparaben exposure is unclear, however some potential explanations include increased islet area due to fragmentation of the islet cluster, augmented beta cell proliferation, larger beta cell size due to greater numbers of cell vacuoles, amplified beta cell differentiation, and increased islet area due to the increased presence of “fuzzy” islets.

Embryos that were exposed to the lowest concentration (250 nM butylparaben) had islet areas that were significantly larger than controls at all time points analyzed (3, 4, 5 and 7 dpf). This could be due to the dispersion of the islet cluster into fragmented cells, where an average of 36% embryos exposed to the 250 nM concentration had fragmented islets over all time points compared to 4% of controls, 7% of embryos exposed to 500 nM, 11% of embryos exposed to 1,000 nM and 3% of embryos exposed to 3,000 nM (Table 3). Another zebrafish study has also found islet dispersion or fragmentation to increase islet area, as seen in embryos that were exposed to 5 nM PCB-126 (Timme-Laragy et al, 2015). Butylparaben-induced cell dispersion has also been shown in rodent models. Alam et al (2013) found butylparaben to cause the collapse of rat Sertoli cell vimentin filaments, allowing spermatogenic cells to become separated from the Sertoli cells and become more dispersed. Butylparaben treatment also increased the size and number of Sertoli cell vacuoles in a time and dose-dependent manner (Alam et al, 2013).
Here, an increased number of vacuoles in beta cells could increase beta cell size and potentially increase islet area.

Greater islet area could also be caused by augmented cell differentiation of beta cell progenitors. Butylparaben has been found to increase cell differentiation, however only in adipocyte cells, demonstrating a limitation of the existing literature. Hu et al (2013) found butylparaben, in the presence of a differentiation cocktail, to increase adipocyte differentiation when compared to cells exposed to DMSO. Parabens have also been found to enhance adipocyte differentiation with increasing alkyl chain length, with butylparaben promoting the most adipocyte differentiation. Taxvig et al (2012) also found butylparaben to promote the differentiation of pre-adipocyte fibroblasts into adipocytes.

The increased incidence of “fuzzy” islets could also be contributing to the larger islet areas observed at the highest concentration (3,000 nM). The cause of this “fuzzy” deformity is still unclear. Butylparaben acts through altering cell membrane properties, and has been shown to allow intercellular solutes to leak from cells by changing cell membrane integrity (Doron et al, 2001 and Furr et al, 1972). It is possible that butylparaben may affect the cell membrane integrity of beta cells in a similar way, causing them to become “leaky” and to appear fuzzy under the microscope. However, if butylparaben were causing the membranes of beta cells to become leaky, it is likely that the membranes of other cells may become leaky as well and appear “fuzzy” under a trans filter. Therefore, further analysis is needed to determine whether the beta cell membranes in these “fuzzy” islets are leaking. Future studies will utilize confocal and histological analysis of these fuzzy islets to examine islet architecture.
It is also possible that the “fuzzy” islets have not aligned properly during development, and have not fully migrated to the right side of the embryo, causing the islets to be visually distorted by the yolk sac. Other environmental chemicals such as PCB’s have been found to interfere with zebrafish islet migration during development. Zebrafish embryos treated with PCB-126 revealed islets that aligned with the third somite rather than the fourth somite between 24 and 48 hpf (Timme-Laragy et al, 2015). Future studies will image embryos that display fuzzy islets ventrally to view islet position and to discover whether butylparaben disrupts islet alignment.

Data from this study also indicate that 250 nM is the lowest concentration in which butylparaben impacts islet development. In this study, the lower concentrations of 62.5 nM and 125 nM appeared to increase islet area in a dose-dependent manner however, this trend was not significant. As a result, because the lowest concentration in which significant pancreatic effects were observed was 250 nM, it is possible that 250 nM (0.0485 mg/L) butylparaben is the LOEC for islet deformities in zebrafish. The lowest concentration in which no significant islet defects occurred was 125 nM, suggesting 125 nM may be the concentration in which no effects are observed, or the NOEC. When exposed to butylparaben daily for 7 days, fatheaded minnows were found to experience a NOEC of 1.0 mg/L butylparaben (Dobbins et al, 2009) and a LOEC of 2.0 mg/L, or 10,297 nM when looking at embryonic growth as an outcome. This is evidence that the pancreas is a more sensitive endpoint in terms of butylparaben exposure.

To further investigate the disrupted islet development, I measured the gene expression of the pancreatic hormone index (insula, gega, sst2 and ghrl) and the
transcription factor *pdx1*. Exposure to butylparaben only significantly decreased the expression of *pdx1* at the highest concentration (3,000 nM), however exposure did not significantly affect the expression of the pancreatic hormone index. Pdx1 plays a role in regulating beta cell function by regulating the expression of insulin. Unlike the hormones of the pancreatic hormone index, Pdx1 is redox sensitive, and can be regulated by the redox state in cells (Rozenfeld *et al*, 2012). In vitro, Pdx1 activity has been found to decrease with the induction of oxidative stress (Matsuoka *et al*, 2007). Additionally, Pdx1 has been found to increase in islets of mice treated with antioxidants N-acetyl cysteine (NAC) and Vitamin C (Kaneto *et al*, 1999). If only oxidative stress were occurring here, it would be expected that *pdx1* gene expression may be affected. However, if Pdx1 activity were to be affected as well, it would likely be reflected in the transcriptional activity of Pdx1, for example, in the transcription of insulin. Because the expression of the pancreatic hormone index does not appear to change in response to the changes *pdx1* expression, it is unclear whether the activity of Pdx1 is affected. Here, the decrease in *pdx1* expression could be a product of oxidative stress, however it is still unclear whether this reduction in *pdx1* expression is contributing to the disrupted islet development that we are seeing at this concentration. Since Pdx1 transcribes for insulin, it would be expected that insulin expression would be downregulated along with *pdx1*. However, here we are seeing no effects on insulin gene expression. It is possible that transcription factors upstream of Pdx1 are being affected. The *forkhead/winged helix* transcription factor (Foxa2) (formerly HNF-3β) is an essential regulator in the development of the foregut, that also plays a role in the beta cell-specific transcription of Pdx1 (Wu *et al*, 1997). It is possible that butylparaben decreases the activity of Foxa2, which would result
in the decreased expression of *pdx1*. Future studies are needed to observe whether butylparaben exposure changes the gene expression of *foxα2*.

The endocrine pancreas can be easily altered by other morphological deformities that occur. In this study, embryonic gross development and the incidence of morphological deformities were accounted for to determine whether butylparaben affects morphological development, and whether these developmental deformities may be causing the altered islet development that we are seeing. Yolk sac utilization and embryonic growth were measured to determine whether butylparaben affects embryonic gross development. In zebrafish embryos, yolk sac area often increases due to an accumulation of edema fluid in the yolk sac, and due to a decrease in absorption of yolk sac nutrients. The yolk sac is mainly comprised of fatty acids, and free fatty acids have been found to regulate the secretion of insulin from beta cells, and have also been found to cause beta cell apoptosis in rat islets, providing a mechanism by which obesity can lead to the development of type 2 diabetes (Itoh *et al.*, 2003 and Cnop *et al.*, 2001). To measure the utilization of nutrients through the yolk sac, embryonic yolk sac areas were measured at 3, 4 and 5 dpf. Due to previous studies that suggest exposure to environmental chemicals inhibit yolk sac utilization in both mouse and zebrafish embryo models, it was originally believed that butylparaben exposure would cause yolk sac areas to increase. DEHP, which is similar to butylparaben because it also activates PPARγ, was seen to significantly inhibit yolk sac utilization in mice in a dose-dependent manner (Sant *et al.*, 2016). Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) significantly increased yolk sac area in exposed zebrafish embryos at 120 hpf (Prasch *et al.*, 2003). Similarly, increasing copper concentrations resulted in significantly larger yolk sacs in
zebrafish embryos at 72 hpf (Johnson et al, 2007). Because no significant trend concerning the effects of butylparaben on yolk sac area was observed, it appears that butylparaben does not significantly affect the utilization of nutrients at the concentrations tested.

Embryo nose-to-tail length was also used as a measurement of gross development. In this study, body length was not significantly affected by butylparaben at any concentration at any time point measured. This may be because the concentrations tested are too low to affect embryo length. When observing the effects of PFOS on zebrafish body length, Shi et al (2008) found that the lower concentrations of 0.1, 0.5 and 1 mg/L PFOS did not significantly affect embryo length at 84 and 96 hpf, however, PFOS significantly inhibited larval growth when exposed to 3 and 5 mg/mL PFOS. As stated above, Dobbins et al (2009), found the LOEC for butylparaben on fat headed minnow growth to be much greater than the concentrations that are tested here (10,297 nM vs. 3,000 nM), suggesting embryonic growth is less sensitive to butylparaben exposure, and the concentrations used in the current experiment are too low to see a significant effect.

The incidence and severity of all deformities were also accounted for in a deformity index. Some morphologic deformities occurred with butylparaben exposure, however most embryos did not have any deformities, and no relationship was observed between developmental deformities and islet architecture, suggesting the pancreas might be a sensitive target tissue of butylparaben. The first exposure assessment of the developmental toxicity of butylparaben in rats found no significant effects on fetal gross development, soft tissue alterations or skeletal alterations at all oral doses tested, with a maximum concentration of 1,000 mg/kg/day (Daston et al, 2004). However, to date, no
studies have been found to exist that account for or quantify the incidence of morphological deformities in response to butylparaben exposure in a fish model. The five deformity outcomes observed in this study are all endpoints that are commonly disrupted by many toxicants in the zebrafish model. Here, the greatest incidence of deformities is seen at the highest concentrations at all time points, however there was no relationship between the occurrence of these deformities and disrupted islet development. Additionally, the lowest concentration (250 nM) did not result in a greater incidence of morphologic deformities when compared to the controls, but did result in significantly greater islet areas and an increased incidence of islet deformities, demonstrating the altered islet development that we are observing is occurring independently of butylparaben-induced deformities, and that the islet might be a sensitive target tissue of butylparaben exposure during development.

There was also no clear trend in the occurrence of specific developmental deformities and deviant islet morphologies. When looking at control, 250 and 3,000 nM treatments, the relationships between specific developmental deformities and islet architecture change based on exposure. For example, pericardial edema appeared to occur independently of islet deformities in the control and 3,000 nM treatment groups (p=0.650 and p=0.739 respectively); however there was a lack of independence in this relationship in the 250 nM treatment group (p=0.005) (Figure 13). This could be a result of the different islet deformities that are prevalent at these two different concentrations: fragmented islets at 250 nM and fuzzy islets at 3,000 nM.
The time of swimbladder inflation is an endpoint that is commonly disrupted by toxicants in zebrafish. Here, the highest concentration of butylparaben (3,000 nM) significantly reduced swim bladder inflation at 4, 5 and 7 days post fertilization.

Exposures to environmental chemicals, such as PCBs have been shown to reduce percent swim bladder inflation. Jonsson et al (2012) saw a concentration-dependent reduction in the number of individual zebrafish embryos that exhibited inflated swim bladders at 4 days post fertilization when exposed to 2 nM PCB 126. The swimbladder is derived from the same endoderm germ layer as the liver, pancreas and the gut. Because the swim bladder and the intestine are both affected by butylparaben, it may be possible that butylparaben affects the developing endoderm. Some toxicants, such as TCDD, have been found to alter both the pancreas and the liver similarly by causing degradation and necrosis of both organs, which may be due to the endodermal origins of both organs (Henry et al, 1997 and McCollum et al, 2011). It is also interesting that the transcription factor for Pdx1 (Foxa2) regulates the development of the foregut that is also derived from the endoderm, and we are seeing a change in the expression of Pdx1 and not in the pancreatic hormone index. This could further support the hypothesis that butylparaben affects the development of the endoderm. Although no apparent liver malformations were observed in this study, because the liver was not an endpoint that was specifically looked at, defects may not be detectible by the imaging methods used.

One way to test for this may be to observe the expression of genes that are required for correct endodermal development such as sox32 (which transcribes for the transcription factor Sox 32 that has been known to inhibit endodermal formation when phosphorylated), sqt, cyc and oep (where mutations in these genes have been found to
lead to failures in endodermal development) as reviewed by Ober et al (2003).

Butylparaben’s effects on endodermal development may also be observed using a $Tg(gut-GFP)$ zebrafish line, in which green fluorescent protein expression is restricted to the endoderm and the organs that it gives rise to. This transgenic line has been used to study both endodermal and pancreatic development (Field et al, 2003).

It is important to note that all butylparaben concentrations used in these experiments aim to represent low-dose and environmentally relevant exposures. Although the exact concentrations of human exposure to butylparaben are still unknown, one study estimates that human exposures due to long-term cosmetic use in the United States ranges from 0.26-14.6 mg/day (Masten et al, 2005) (Table 4). Multiple studies have observed post-exposure concentrations of un-metabolized butylparaben in human urine (0.2-1,240 µg/L), blood (135 µg/L) and amniotic fluid (0.3 µg/L) (Table 4). Because the majority of butylparaben is metabolized upon exposure, it is likely that these concentrations are lower than the actual human exposures. In these studies, zebrafish embryos were exposed to butylparaben concentrations that range from 48.5-582.66 µg/L (250-3,000 nM), and because these concentrations are within the ranges found in human samples, it is likely that the concentrations used in these studies are much lower than those of human exposure.

Table 4. A comparison of pre and post-metabolism butylparaben concentrations in humans and in the concentrations used in these studies.

<table>
<thead>
<tr>
<th></th>
<th>Pre-metabolism Exposure</th>
<th>Post-metabolism measure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Humans</strong></td>
<td>260 – 14,600 µg/day</td>
<td>Urine: 0.2-1,240 µg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amniotic Fluid: 0.3 µg/L</td>
</tr>
<tr>
<td><strong>Zebrafish</strong></td>
<td>48.5-582.6 µg/L</td>
<td>?</td>
</tr>
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</table>

(Calafat et al, 2010)  
(Janjua et al, 2007)   
(Philippat et al, 2013)
Although all methods in this study have been performed successfully, there are limitations to some of the methods. Islets are often occluded by pigmentation at 3 and 4 dpf, which can make it difficult to obtain a clear view of the islet while imaging. Islets are also often imaged through the yolk sac until 5 dpf and can therefore also become distorted by the yolk sac contents. An alternative method that could be used to assess whether butylparaben affects islet development is flow cytometric analysis. Flow cytometric analysis has been performed to count green fluorescent protein labeled beta cells from mouse islets (Hara et al., 2003). Flow cytometry would count the exact number of beta cells in the pancreatic islets, and also observe if exposure to butylparaben is increasing or decreasing islet area due to changes in the numbers of beta cells. Imaging with a confocal microscope would also allow for a three-dimensional image of the islet to be obtained, allowing for more accurate measurements of islet areas.

This study demonstrates the effects of butylparaben on the development of the pancreatic islet as indicated by an increase in islet area and the incidence of islet deformities. Butylparaben does not however appear to affect embryonic morphologic growth, and there was no association between the incidence in morphologic deformities and islet architecture, suggesting the pancreatic islet may be specifically targeted by butylparaben. However, it is still unclear whether any of these developmental effects translate to functional consequences, and future studies are needed to characterize the long-term effects of these exposures.
CHAPTER 3

BUTYLPARABEN DISRUPTS REDOX BALANCE IN THE DEVELOPING EMBRYO

3.1. Introduction

Embryonic development is extremely sensitive to oxidative stress due to changes in cell proliferation, development and differentiation that take place during this life stage. Reactive oxygen species (ROS) play an important role in normal embryonic development by orchestrating cell signal transduction, cell-fate decisions and apoptosis (Ufer et al., 2010). Embryonic exposure to environmental chemicals, such as parabens, can generate ROS and cause disruptions in cellular redox status (Hansen et al., 2006). Embryonic oxidative stress can lead to carcinogenesis, cardiovascular dysfunction, pulmonary diseases, neurodegenerative diseases and diabetes (Wells et al., 2009). Consequently, antioxidant functions that defend against oxidative stress during embryonic development are essential.

The most abundant endogenous antioxidant is glutathione (GSH), a tripeptide that consists of glutamate, cysteine, and glycine. GSH acts as an antioxidant by scavenging ROS that oxidize the thiol group of the cysteine residue. When GSH is oxidized, it can dimerize to form glutathione disulfide (GSSG), which can then be recycled back into GSH by the enzyme glutathione disulfide reductase (Gsr), in a reaction that requires NADPH (Hansen et al., 2006). The synthesis of GSH involves two ATP-dependent enzymatic steps. In the rate-limiting first step, glutamate-cysteine ligase (Gcl) catalyzes the combination of glutamate and cysteine, and next glutathione synthetase (Gss) adds glycine to the glutamate-cysteine molecule (Timme-Laragy et al., 2013).
Butylparaben exposure has been found to be linked to the occurrence of oxidative stress in rodent models (Shah et al, 2012 and Hegazy et al, 2015), and has been associated with biomarkers of oxidative stress in humans (Kang et al, 2013 and Watkins et al, 2015). This study aims to analyze whether daily exposure to butylparaben results in the occurrence of oxidative stress. Studies measured the redox potentials of GSH and Cysteine, along with total GSH and total Cysteine. Gene expression was also conducted on the enzymes that are responsible for GSH synthesis (Gclc, Gclm and Gss), and on the enzyme responsible for recycling oxidized GSSG back into reduced GSH (Gsr). The expression of two GST enzymes (Gstp and Gsta1) was also measured. These studies aim to determine whether exposure to butylparaben affects glutathione redox dynamics, and whether it can be associated with the occurrence of oxidative stress in the developing zebrafish embryo.

3.2. Materials and Methods
3.2.1. Zebrafish Husbandry

Tg (ins-GFP) embryos were obtained from Dr. Phillip diIorio at the University of Massachusetts Medical School zebrafish facility (Worchester, MA), and the heterozygous nrf2a<sup>fh318</sup> embryos were crossed to obtain the wildtype nrf2a<sup>+</sup>+ used in the gene expression experiments. This genetic line was generated by the Tilling Mutagenesis Project, obtained from the Moens Laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA) and was acquired as a generous gift from Dr. Mark Hann of the Woods Hole Oceanographitic Institute (Woods Hole, MA). All zebrafish were maintained as described above in chapter 2.2.2.
3.2.2. HPLC Analysis of Soluble Thiols

Embryos were collected from group matings in three 6 liter tanks of an approximate 1:2 male to female ratio. Embryos were stored at 28.5 ºC and maintained at low densities (30 embryos per 10 mL) in 0.3x Danieau’s solution and were exposed daily to 0.01% (v/v) DMSO, 500 or 1,000 nM butylparaben. Embryos were collected at 24 hpf, or re-exposed to butylparaben at 24 hpf and collected at 28 hpf. All embryos were placed in 5% PCA buffer and samples were then immediately stored at -80ºC. Approximately 60 embryos were used in each treatment.

GSH, Cysteine, GSSG, Cystine, total GSH, total cysteine and the GSH and cysteine redox potentials (Eh) were measured at the University of Michigan, Ann Arbor, Michigan by Dr. Karilyn E. Sant using High-performance Liquid Chromatography (HPLC). As described by Sant et al (2016) a Waters 2695 Allicance Separations module with a Supelcosi LC-NH2 column was paired with at Waters 2475 Fluorescense Detector (Sigma, St. Louis MO). Reverse phase chromatography measured GSH, GSSG, cysteine and cystine. The flow was set to 1 mL/min using mobile phase A (80% methanol) and mobile phase B (62.5% methanol, 12.5 glacial acetic acid and 214 mg/mL sodium acetate tryhydtrate). Fluorescent detection (excitation 335 nm, emission 518 nm) allowed peaks to be visualized, which were then processed with a Waters Empower software (Milford, MA). The Nernst equation \( V_{\text{Eq}} = (RT/zF) \ln([X_{\text{out}}]/[X_{\text{in}}]) \) was used to calculate the GSH and Cysteine redox potentials.

3.2.3. Quantitative real-time PCR

The expression of glutathione-related genes: the catalytic and modifier subunits of Gcl (gclc and gclm), glutathione synthetase (gss), glutathione disulfide reductase (gsr),
and two glutathione-s-transferase enzymes (gstp1 and gsta1) were measured using quantitative real-time PCR with RNA extraction and reverse transcription, which were performed as described previously in chapter 2.2.8. All primers were first tested for specificity and efficiency. Primers were designed using Primer-BLAST (NCBI) and were optimized for melting temperature and for dimerization, to avoid both homodimer and heterodimer dimerization. Quantitative real-time PCR was also performed as described in chapter 2.2.9, with β-actin utilized as a housekeeping gene. The primer sequences (5’-3’ orientation) used for the genes gclc, gstp, gsta1 and housekeeping gene β-actin are listed in Table 5. Primers for gclm, gss, and gsr were purchased from Bio-Rad (PrimePCR™).

Data obtained from qPCR were analyzed using the Bio-Rad CFX Connect Manager™ software, version 3.0 (Bio-Rad). The ddCT method was used to calculate fold change.

Table 5. Primer sequences of glutathione-related genes gclc, gstp, gsta1 and housekeeping gene β-actin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 5’-3’</th>
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<tbody>
<tr>
<td>gclc</td>
<td>Foreword AACCGACACCCAAGATTCAGCCT</td>
</tr>
<tr>
<td>gstp</td>
<td>Foreword CGACTTGAAAGCCACCTGTGC</td>
</tr>
<tr>
<td>gsta1</td>
<td>Foreword TCACACCTGCCGAAAACAA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Foreword CAACAGAGAGAAGATGACAC</td>
</tr>
</tbody>
</table>

3.3.4. Statistical Analysis

Data was analyzed using Microsoft Excel and Stata statistical software, version 14.1. All statistical tests were performed by Stata. Two-factorial ANOVAS followed by a Tukey’s post hoc test or a pairwise t-test were used to test for effects among exposed
groups and controls. Data are presented as mean and the standard error of the mean (SEM).

3.3 Results

3.3.1. Analysis of Glutathione and Cysteine Redox Potentials

To analyze redox status in the embryos, GSH, Cysteine, GSSG, Cystine, total GSH, total cysteine and the GSH and cysteine redox potentials ($E_h$) were measured using High-performance Liquid Chromatography (HPLC), with the Nernst equation used to calculate the GSH and Cysteine redox potentials. There were no significant changes in the redox status of embryos that were exposed to butylparaben at 3 hpf and were collected at 24 hpf (Figure 15.B). However, significant changes in redox status were observed in embryos that were exposed to butylparaben at 3 and 24 hpf and collected at 28 hpf. Butylparaben concentrations of 500 and 1,000 nM increased GSH by 10% and 40%, respectively, and decreased GSSG by 37% and 59%. GSH redox potentials became more reduced with 500 nM and 1,000 nM butylparaben exposures, decreasing redox potentials by 7 and 18 mV, respectively. Cysteine redox potentials also became more reduced, decreasing by 17 and 28 mV (Figure 15.A.). Total GSH and total cysteine did not significantly change with increasing concentration.
Figure 15. Redox analysis of glutathione and cysteine at 28 hpf and 24 hpf. A. Redox analysis of glutathione and cysteine at 28 hpf. Embryonic reduced and oxidized glutathione (GSH, GSSG) and cysteine were quantified at 28 hpf after exposure to DMSO, 500 nM or 1,000 nM butylparaben at 3 hpf and 24 hpf. The Nernst equation was used to calculate cellular redox potentials (Eh). Two factor ANOVAS were used to determine differences between exposed and control groups followed by a Tukey’s post hoc test (p<0.05, n= 3 samples of 20 pooled embryos). B. Redox analysis of glutathione and cysteine at 24 hpf. Embryonic reduced and oxidized glutathione (GSH, GSSG) and
cysteine were quantified at 24 hpf after exposure to DMSO, 500 nM or 1,000 nM butylparaben at 3 hpf. The Nernst equation was used to calculate cellular redox potentials (Eh). Two factor ANOVAs were used to determine differences between exposed and control groups followed by a Tukey’s post hoc test (p<0.05, n= 1-3 samples of 20 pooled embryos).

Figure 16. Gene expression of glutathione-related genes. Wildtype embryos were exposed daily to DMSO, 250, 500 or 3,000 nM butylparaben, and were collected at 78 hpf. Expression of gsta1, gccl, gss and gsr was measured and the ddCt method was used to calculate fold change, followed by pairwise t-tests to calculate significance (p< 0.05). N= 5 biological replicates per group with each replicate containing pools of 10-13 embryos.
3.3.2. Expression of Glutathione-related Genes

The expression of glutathione-related genes was also measured to indicate the occurrence of oxidative stress. Gene expression of enzymes that are involved in glutathione synthesis: genes encoding for the two subunits of glutamate-cysteine ligase (gclc and gclm) and glutathione synthase (gss) were measured in addition to glutathione disulfide reductase (gsr), that recycles oxidized glutathione (GSSG) back into reduced glutathione (GSH). The expressions of two genes that encode for glutathione-S-transferases (gstp and gsta1) were also measured. No significant relationship was found between butylparaben exposure and the expression of gclm. However, gclc, gss and gsta1 were all significantly downregulated at the highest concentration (3,000 nM) whereas gsr was significantly upregulated at all concentrations in a dose-dependent manner (Figure 16). The expression of gstp was upregulated at the 500 nM concentration (Figure 16).

3.4. Discussion

Oxidative stress occurs when there is an increase in reactive oxygen species (ROS) and insufficient antioxidant defenses, which often leads to toxic effects such as damage to lipids, proteins and nucleic acids, contributing to many diseases, including diabetes. Antioxidants such as glutathione (GSH) and cysteine defend against oxidative stress by scavenging ROS. However, reactive oxygen species do play important roles in embryonic development. The redox status, or the ratio of oxidized to reduced antioxidants, plays a major role in cell signaling and cell fate decisions. More oxidized redox states often signal cell apoptosis whereas more reduced states lead to cell proliferation.
In this study, the redox potentials of the thiol-containing redox couples glutathione and cysteine were measured in response to butylparaben exposure in 24 hpf and 28 hpf zebrafish embryos. Significant changes in redox status were only seen in embryos that underwent an acute exposure before collection (exposed at 24 hpf and collected at 28 hpf). Embryos that were exposed to butylparaben 4 hours before collection saw significant changes in both glutathione and cysteine redox potentials (Figure 15.A.), whereas embryos that were exposed 24 hours before collection saw no significant changes (Figure 15.B).

Cysteine is regulated independently of GSH and sits at a more reduced state (Jones et al, 2006). If butylparaben were to cause oxidative stress, it would be expected that the GSH and cysteine redox potentials would become more oxidized with increasing butylparaben concentration. However, in this study, reduced glutathione (GSH) and cysteine increased with increasing butylparaben concentration, while oxidized glutathione (GSSG) and cystine decreased with increasing concentration, resulting in a more reduced redox state (Figure 1).

These results contrast somewhat with what has been reported in the literature. Shah et al (2012) found butylparaben decreased GSH by 43.8% in the liver tissues of rats that were given 1,320 mg/kg bw/day butylparaben. Hegazy et al (2015) treated rat dams orally and subcutaneously with 200 mg/kg bw/day butylparaben and measured GSH dynamics in the brain tissues of the developmentally exposed offspring. Both oral and subcutaneous butylparaben treatments were found to significantly decrease GSH and the GSH/GSSG percent ratio, and to significantly increase GSSG and percent oxidized glutathione, suggesting oxidative stress occurrence in the brain tissues of the offspring.
None of these studies however observe the effects of butylparaben in the developing zebrafish model, and butylparaben concentrations used in the animal studies described above are much higher than the concentrations used in the current study. Therefore, it is possible that butylparaben causes reductive conditions in the developing zebrafish, or at lower concentrations, and results in oxidized conditions in mammalian models or at higher concentrations; or it is possible that we are capturing a rebound effect. The animal studies that investigate butylparaben and oxidative stress parameters also only examine specific organs such as liver and brain tissues, whereas this study analyzes the entire embryo. Therefore, it is possible that butylparaben exposure results in oxidized conditions in some organs, and reductive conditions in others.

When reductive conditions are detrimental, they are often referred to as “reductive stress.” Reductive stress is defined as “an abnormal increase in reducing elements” such as an increased ratio of reduced to oxidized glutathione (Zhang et al, 2010). Reductive stress may alter cell proliferation and has been found to delay protein folding in yeast, and can lead to protein aggregation, resulting in cardiomyopathy in mice (Simons et al, 1995 and Zhang et al, 2010). Because the redox results in this study show increased ratios of reduced to oxidized glutathione and cysteine, it is possible that reductive stress is occurring here. However, due to the studies that show that butylparaben causes oxidative stress, it is also possible that the embryos are able to recover and upregulate antioxidant defenses, in which case we may be missing the window where oxidative stress occurs. When oxidative stress first occurs due to an accumulation of ROS, antioxidant defenses are initially slightly depleted and are then turned on in an adaptive response. As ROS continue to accumulate, antioxidant defenses become depleted and fall
below baseline in a toxic response (Figure 17). In these studies, increasing GSH with decreasing GSSG along with the decreased expression of GSH-related genes may indicate that redox and gene expression were analyzed when embryos were undergoing an adaptive response.

![Graph showing GSH and ROS levels](image)

**Figure 17.** Increasing levels of reduced glutathione may indicate the occurrence of an adaptive response.

In order to further explore whether oxidative stress occurs with butylparaben exposure, the expression of genes that are involved in the synthesis of glutathione were measured. Glutathione is a tripeptide that is synthesized via two ATP-dependent steps. The first step is rate-limiting and occurs when the enzyme glutamate–cysteine ligase (Gcl) combines a glutamate and cysteine molecule. Gcl is composed of a catalytic subunit (Gclc) and a modifier subunit (Gclm) that is responsible for controlling the rate of GSH synthesis, and the expression of both subunits have been shown to increase or decrease in
response to reactive oxygen species (Timme-Laragy et al., 2013, Huang et al., 1993 and Dickinson et al., 2004). Gclc is responsible for the catalytic activity of Gcl and can be directly inhibited by GSH, whereas Gclm does not possess any catalytic function but regulates the activity of Gclc (Dickinson et al., 2004). Oxidative stress is known to enhance Gcl activity and increase Gcl expression (Franklin et al., 2009). Therefore, if oxidative stress were to occur, the expression of gclc and gelm would be expected to increase. The gene expression of both gclc and gclm were measured and showed that only the expression of gclc was affected by butylparaben exposure. Embryos exposed to the highest butylparaben concentration (3,000 nM) had significantly decreased expression of gclc when compared to controls.

During the second step of GSH synthesis, the enzyme glutathione synthase (Gss) adds a glycine to the glutamate-cysteine molecule. We also measured the expression of gss and found that its expression was concordant with the expression of gclc, and was also significantly downregulated with exposure to 3,000 nM butylparaben. The decreased expression of both gclc and gss suggest decreased activity of both the Gcl and Gss enzymes. The decreased activity of both Gcl and Gss would likely result in less GSH synthesis. Piao et al. (2011) found that exposure to silver nanoparticles inhibited the activity of Gclc and Gss in human liver cells, leading to decreased GSH levels and an increase in ROS. However, because total glutathione was not significantly affected by butylparaben exposure, this decreased expression cannot be directly translated to decreased Gcl and Gss enzymatic function. Here, it is unclear whether the reduced expression of gclc and gss indicate the occurrence of oxidative stress.
To further investigate whether butylparaben causes oxidative stress in the developing zebrafish, the expression of glutathione disulfide reductase (Gsr) was also measured, which is responsible for recycling oxidized GSSG back into reduced GSH in a NADPH-dependent reaction essential for maintaining GHS levels. We found that the expression of gsr significantly increased at all concentrations analyzed (250, 500 and 3,000 nM). Redox analysis also showed that GSSG decreased with increasing GSH in a dose-dependent manner. Because gsr expression was upregulated at concentrations that were also analyzed for redox status, it is possible that Gsr enzymatic activity is also increasing. Increased Gsr activity would recycle greater amounts of GSSG back into GSH, possibly resulting in less GSSG and more GSH. This increase in Gsr activity may be causing the increased levels of reduced GSH and decreased levels GSSG that are seen in the redox analysis data. These results also further suggest a reduced environment with butylparaben exposure, and because gene expression was measured two days after redox potentials were measured, it appears these reductive effects are lasting.

In contrast to our findings, Shah et al (2011) found butylparaben exposure to decrease Gsr levels and Gsr activity in a dose-dependent manner in liver tissues of mice, indicating the occurrence of oxidative stress. The concentrations used by Shah et al however were much greater than the concentrations used in this study, ranging from 13.33 to 40 mg/0.2mL olive oil/kg bw/day. Therefore, the concentrations used in our study may not be great enough to decrease Gsr activity or affect its expression. Additionally, experiments by Shah et al were carried out in mice, which are a different animal model than the zebrafish model used here, and only the liver tissues of these mice were analyzed whereas our study analyzes whole embryos. It is possible that Gsr activity
increases in the liver but decreases in other tissues, an outcome that may be occluded in our experiments. Experiments performed by Shah et al also analyzed Gsr concentrations and activity whereas here, only the gene expression of gsr was measured. Because of these differences it is difficult to compare these two studies.

The mechanism that is causing the upregulation of gsr is still unclear. Unlike the enzymes that are involved in GSH synthesis, Gsr requires NADPH to recycle GSSG back into GSH; therefore it is possible that exposure to butylparaben results in the upregulation of NADPH. The activity of Gsr can be monitored by NADPH consumption, and because NADPH fluoresces at excitation/emission wavelengths of 340/460 nm (Smith et al, 1988), future studies could analyze whole-embryo NADPH concentrations by measuring its fluorescence. The pentos phosphate pathway is a major source of NADPH and is a major source of reducing agents (such as NADPH) for biosynthetic processes and maintaining redox potentials to protect against oxidative stress (Juhnke et al, 1996). Therefore it is possible that this pathway is affected by butylparaben exposure, and is resulting in increased levels of NADPH.

In addition to NADPH, Gsr also requires flavin adenine dinucleotide (FAD) as a redox cofactor for its activity. FAD has been seen to stimulate Gsr activity in vitro, and small amounts of 1 µM FAD can activate Gsr within 10 min (Beutler et al, 1969). Because Gsr is especially sensitive to FAD, it is also possible that exposure to butylparaben up-regulates or increases FAD in the embryos.

Glutathione also defends against oxidative stress by acting as a cofactor for the family of enzymes: glutathione-S-transferases (GSTs). This enzyme family is responsible for the detoxification of many xenobiotics by conjugation to GSH. The conjugated
xenobiotic metabolites are often more hydrophilic than their unconjugated parent molecules, and therefore more easily excreted from cells, making them less toxic (Garner et al, 2012). Three classes of GST enzymes (Alpha, Pi and Mu classes), and 19 GST genes that are responsible for metabolizing xenobiotics, have been identified in the zebrafish model (Timme-Laragy et al, 2013). Here, gene expression was analyzed for gsta1 and gstp1. Similar to the expression of gclc and gss, gsta1 was significantly downregulated at the 3,000 nM concentration. However, gstp1 was significantly upregulated at the 500 nM concentration. Shah et al (2011) also found butylparaben exposure to significantly reduce GST activity in the liver tissues of exposed mice, suggesting that this reduction in activities of these enzymes may be due to butylparaben-induced protein oxidation. Because butylparaben was also found to cause lipid peroxidation, the products of lipid peroxidation with these enzymes could modify their histidine residues, generating protein-protein crosslinked products, reducing their enzymatic activities (Shah et al, 2011). In this study, the downregulation of Gsta1 is congruent with the gene expression results and with the redox results that are described above, suggesting butylparaben exposure results in a more reduced state. However, the upregulation of Gstp1 suggests a more oxidized state.

This study suggests butylparaben exposure results in a more reduced redox state in the developing zebrafish. Gene expression of enzymes involved in glutathione synthesis, the catalytic subunit of Gcl (gclc) and gss were significantly downregulated at the highest concentration. However, total glutathione was not significantly affected. This could be because the expression of gclc and gss is downregulated, but the enzymatic functions are not being affected. Additionally, redox analysis was not performed in
embryos that were exposed to the highest concentration (3,000 nM butylparaben). The expression of $gsr$, that recycles GSSG back into reduced glutathione (GSH), was significantly increased at all concentrations, and these concentrations were also analyzed for redox analysis. Redox data showed that GSSG decreased while GSH increased in a dose-dependent manner. This could be due to the increased expression of $gsr$, suggesting the enzymatic activity of Gsr is increasing with butylparaben exposure. Therefore, it cannot be concluded whether GSH synthesis is affected by these concentrations of butylparaben, however, it appears that butylparaben exposure results in a more reduced redox state. These results do contradict other studies that show butylparaben causes oxidative stress, though none of these studies use the zebrafish model, and the concentrations used in other animal studies are much higher than those used in this study. Although it is still unclear whether exposure to butylparaben causes reductive or oxidative stress, the data from this study indicate that exposure to butylparaben perturbs the redox status in the developing embryo. Future studies need to be conducted to further determine whether butylparaben causes oxidative stress in the developing zebrafish.
CHAPTER 4
CONCLUSIONS

These studies reveal that embryonic exposures to butylparaben affect pancreatic development in the zebrafish model, however, there is not enough evidence to conclude whether oxidative stress is involved. Exposure to butylparaben results in increased islet area, and an increased incidence in islet deformities while not significantly affecting embryonic gross development. There is also no apparent association between morphological deformities and islet architecture, suggesting the islet may be especially sensitive to butylparaben as noted earlier. Butylparaben also appears to create a more reduced redox environment. Reduced GSH and cystine increase with decreasing oxidized GSSG and cysteine in a dose-dependent manner, and both glutathione and cystine redox potentials become more reduced with increasing concentration. This could indicate increased enzymatic activity of Gsr, that is responsible for recycling GSSG back into GSH. The gene expression of the enzymes involved in the synthesis of glutathione (gclc and gss) are significantly downregulated at the highest concentration, however total glutathione is not significantly affected by butylparaben, suggesting the activity of these enzymes may not be changed. The increasing reduced glutathione with increasing butylparaben concentration, along with the down-regulation of glutathione-related genes may indicate that embryos were analyzed while undergoing an adaptive response to oxidative stress.

It is still unclear whether oxidative stress is the mechanism that is driving the development of these deviant islet morphologies, and it is possible that another mechanism is contributing to this such as PPARγ activation. Butylparaben has been seen to activate PPARγ, resulting in increased adipocyte differentiation. Although it is unclear
what is contributing to the increased islet areas observed in these studies, PPARγ-activated beta cell differentiation may potentially play a role.

To further investigate whether butylparaben induces oxidative or reductive stress, and whether this stress may impact the developing islet, future studies will analyze pancreas development with antioxidant N-acetylcysteine (NAC) and pro-oxidant tert-butyl hydroperoxide co-treatments. RNA sequencing also needs to be performed in order to determine whether other oxidative stress related genes are being affected along with other genes that are involved in pancreatic development.

It is still unclear whether these islet deformities will develop into functional consequences, or if embryos are able to recover once the chemical exposure has been removed. Further studies are needed in adult zebrafish that were exposed to butylparaben as embryos, to see whether these islet deformities lead to insulin resistance later in life, and potentially the development of diabetes. The zebrafish is an ideal model to test for this because the diabetic zebrafish displays known secondary complications that are associated with diabetes in humans such as neuropathy, and impaired wound healing, which can be measured by caudal fin regeneration (Intine et al., 2013). Additionally, the zebrafish can regenerate its damaged pancreas and restore it to a similar state that would be expected in a post-transplant human patient (Intine et al., 2013). Analysis of glucose homeostasis can also be performed in adult zebrafish via blood analysis.

However, very little is still known about the impact of environmental toxicant exposures on the developing pancreas and how oxidative stress impacts development. The results from these studies aim to contribute to the understanding of how exposure to an environmental toxicant, butylparaben, affects the developing pancreas. Future studies
are needed to investigate and whether oxidative stress is a mechanism that may impact
the pancreas and lead to the development of disease, specifically diabetes, later in life.
BIBLIOGRAPHY


