POPULATION VARIABILITY AND THE TERATOGENIC EFFECTS OF DIOXIN DURING PREGNANCY

A Dissertation

by

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ABSTRACT

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) during pregnancy is of particular concern as it interrupts fetal growth and development. While susceptibility to many toxicants often varies among individuals due to genetic differences, current studies of the teratogenic effects of dioxin do not account for inter-individual variability when evaluating exposure risks. Our study aims to evaluate the effects of dioxin exposure on pregnant females and fetal development in genetically diverse mice to determine how genetic background impacts susceptibility. We developed an *in vivo* study with a panel of mice that collectively mimic a heterogeneous human population. In this study, pregnant female mice from 16 diverse mouse strains are exposed to one of three doses of dioxin (0, 1, 100 ng/kg/day) for a period of 10 days following mating. At E10.5 (post-mating) mice are euthanized and embryos dissected. To determine effects of dioxin at different doses, non-cancerous pregnancy-related and cardiogenic endpoints were assessed. We found that genetic background influences response variation in both a strain- and endpoint-dependent manner. This data emphasizes the importance of accounting for genetic background when studying the effects of toxic chemical compounds and individual susceptibility.

DEDICATION

I would like to dedicate this to my daughter, Cadey, and nephews, Ayden and Joshua.

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I.1 Risk Assessment of Environmental Toxicants

I.1.1 Introduction

For many years, federal and state government agencies, including U.S. Environmental Protection Agency (US EPA), have used risk assessment as the primary tool in addressing the concerns of various hazardous exposures(Abt, Rodricks, Levy, Zeise, & Burke, 2010). This process is formally involved in “planning risk assessment and ensuring thoroughness consistent with decision-making”(Abt et al., 2010). Human risk assessment guidelines generally consist of four essential steps: (1) hazard identification, (2) dose-response assessment, (3) exposure assessment, and (4) risk characterization(Clewell, 2005; Health, 1983) (Figure 1-1).

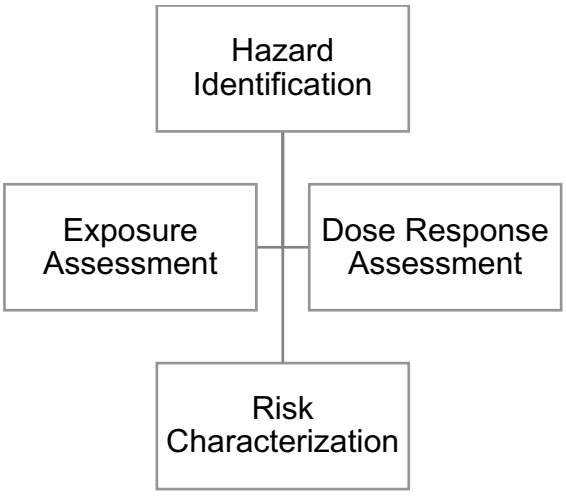


Figure 1-1. U.S. Environmental Protection Agency (EPA) 4-Step Risk Assessment Process.

I.1.2 Hazard Identification

The goal of hazard identification is to identify chemical substances that pose a threat to the overall well-being to the general population. During this step, researchers must first identify the toxic compound and where the primary exposure occurs(Gundert-Remy & Sonich-Mullin, 2002). It must then be determined which individuals within the general population that experience adverse effects and how they are exposed. With this information, scientist move forward to the exposure and dose response assessments.

I.1.3 Dose Response & Exposure Assessment

Dose-response assessment is key to quantifying adverse exposure outcomes and the likelihood these outcomes occur at various exposure levels(Chiu & Slob, 2015). Recent advances in developing these guidelines help quantify response variability and uncertainties, however, results greatly differ among endpoints(Chiu & Slob, 2015). Toxicodynamics (the effects of toxic compounds in the body) and toxicokinetics (the biological absorption and distribution leading to toxicity) are key components of hazard analysis and are critical for determining the mode-of-action (MOA) of a specific toxicant(Gundert-Remy & Sonich-Mullin, 2002). Starting with the active form of the toxic compound, this sequence of events leads to an observed response used to identify influential biological elements of dose-response(Clewell, 2005). The MOA serves as proof of a response threshold, signifying the existence of upstream biochemical responses(Crump, 2011). Response curves are generated primarily from laboratory animal data and generally have two shapes: linear or nonlinear (i.e. sigmoidal)(Crump,

2011). Linear curves do not have inherent thresholds and imply that toxic response is capable at all levels of exposure. Nonlinear curves utilize the MOA and uncertainties in response found in data to formulate inherent thresholds in the response curves.

Exposure assessment, integrates both hazard identification and dose-response to measure the intensity, prevalence, distribution and reoccurrence of the target compound. The exposed community are assessed through health questionnaires and evaluations during exposure assessment, and the magnitude and frequency of the affected population are measured(Peters, Glass, Milne, Fritschi1, & consortium, 2014). Some individuals respond to chemical hazards more aggressively than others, and this increases the uncertainty in calculating a population response(Chiu & Slob, 2015). This data helps to identify the level of uncertainty associated with exposure risks and susceptibility to toxic chemical substances.

I.1.4 Risk Characterization

The final step, risk characterization, involves integration of the all the data collected from previous steps and the key findings and uncertainties are used in making final decisions on the overall risks associated with the toxic compound(Chiu & Slob, 2015). Risk characterization utilizes all the steps pf risk assessment to illustrate how the population will be affected by the target compound(Health, 1983; Jardine et al., 2003). With an array of risk assessment tools and data, the assessor makes decisions on how to assess the risks at hand. They also determine the amount of uncertainties associated with the data

and determine the final decisions that are made concerning health policy and exposure prevention(Jardine et al., 2003; Walton, Dorne, & Renwick, 2001).

If models lack sufficient knowledge of toxicity mechanisms, confounding health factors, or other toxicity-related factors, then risk estimations become largely contingent upon linearity within the low-dose (or “unobservable”) region of dose-response curves(Crump, 1996; Gaylor, Kodell, Chen, & Krewski, 1999). Dose response curves are generated and analyzed further to predict safe exposure levels and response curves that lack thresholds undergo linear extrapolation. This method predicts the lowest risks for an adverse response, including negligible responses found within the low dose region(Gaylor et al., 1999).

I.1.5 Individual and Population Response

Current risk assessment procedures analyze for a range of responses caused by chemical compounds. Response variability can be due to a wide array of extrinsic (i.e. diet, environmental co-factors, etc.) and intrinsic factors (i.e. genetic disposition, age, disease-state, etc.). Individual human health responses vary substantially in their response to toxic chemical compounds and this should be quantitatively integrated into risk decisions. Response due to genetic variability is assumed not to exist below the threshold, but there are highly susceptible backgrounds that can indeed fall below calculated response thresholds. Such factors account for small subgroups of the exposed population that are statistically insignificant when aggregated in current risk analyses. Population response is quantitatively assessed through the use of standard uncertainty

and threshold factors that do not account for individual variability. In such cases, those highly susceptible individuals tend to fall below the RfD (into the nonlinear low-dose range) and fall within the dose range that predicts no adverse outcomes. Uncertainty and genetic variability greatly impact all aspects of risk assessment but has not yet been fully recognized.

The Hokkaido study, is just one example of the importance of understanding the effects of early low-dose environmental chemical exposures (i.e. dioxins, pesticides, methylmercury, etc.) on health outcomes of genetically susceptible subpopulations of children(Kishi et al., 2017). Other studies, much like the Chapaevsk (Russia) cohort study, have identified susceptible subpopulations (i.e. environmentally exposed adolescent boys) by identifying predictors of toxicity to chemical compounds, such as dioxins and dibenzofurans(Hauser, 2005). Regulatory agencies require more evidence to integrate these data into risk guidelines, but there is simply not enough information on how interindividual variability influences the risks of non-cancer effects(Bogen, 2016).

Not all non-cancer outcomes have a threshold or non-linear response, due to background diseases and genetic influences found within the general population. For example, exposure levels that fall below the RfD are assumed “safe”, but lack further analysis or evidence(Chiu & Slob, 2015). In these cases, the RfD serves with limited function and can lead to misinterpretation of low-dose exposure risks. Emphasis on a unified approach for assessing safe exposure levels to cancerous and non-cancerous toxicants has long been argued(Bogdanffy et al., 2001; Calabrese & Baldwin, 2001; Chiu & Slob, 2015;

Crump, 2011; Gaylor et al., 1999). This approach relies on increased knowledge of toxic mechanisms and serves to address the uncertainties of human exposures within the low-dose (unobservable) regions of dose-response curves(Crump, 1996). A unified framework will strengthen dose-response assessment by increasing step-wise precision and transparency of the process(Chiu & Slob, 2015). New methods of risk assessment are especially crucial for highly susceptible individuals within the population. In order to address all health concerns, we must utilize a technique that encompasses genetic variability as the driving force in understanding dose response.

The human response to toxic chemicals varies in both magnitude of effects and susceptibility. A better understanding of how genetics influences individual response and susceptibility could link chemical assessment paradigms to more precise exposure risks calculations. With this knowledge a more accurate population-level approach can be used to predict toxicity in areas under current investigaton(Harrill & McAllister, 2017).

I.1.6 Applications of Mouse Models for Risk Assessment

The underlying goal of toxicology is to understand the toxic mechanisms of chemical compounds and the risks for adverse health effects. The overall decision-making process becomes increasingly difficult to accomplish when needed information from human data is ethically limited. One such study found human data on the endocrine disrupting effects of dioxin to be difficult to ascertain; so, the study utilized organ-on-chip technologies, along with murine models to assess the endocrine disrupting risks of dioxin exposure in women(Bruner-Tran et al., 2017). This study, like many others, used translational means

of assessing risks with the knowledge that human exposure data cannot be generated at will and is only available through accidental exposures. Also, the limited human data available which can be complicated by co-exposures to other toxicants and uncertainties in determining the exposure dose.

For many decades, mouse models have provided substantial contributions to understanding human exposures to chemicals ranging from pharmaceutical compound to environmental contaminant assessments(Grassman, Masten, Walker, & Lucier, 1998). The use of mice limit the dangers and ethical constructs faced when using humans in clinical studies(J, M, & M, 2014). Although the mouse genome does not exactly match that of a human, it is very easy to manipulate and offers broad variations in genetic background. Mice can also exhibit more rapid reproduction times compared to humans, which is beneficial when specific genetic background are needed. The mouse model can serve as a surrogate response for humans, in which both human and animal *in vivo* data are analogous(Grassman et al., 1998; Portier, 2001).

The human genome varies widely across individuals; uniquely, the mouse genome can be manipulated to resemble more genetic variability than observed in the human population. A recent study conducted utilized a diverse mouse panel (known as the collaborative cross mice) to understand how genetic background influences response variation to a murine virus in understanding neurological diseases (i.e. Theiler's murine encephalomyelitis virus)(Brinkmeyer-Langford et al., 2017). Another study uncovers similar response variation in diverse murine populations by demonstrating the

toxicological relationship between perchloroethylene and trichloroacetate(Cichocki, 2017). These data, and similar studies, utilize the advantages of using diverse mouse models as a resource for understanding human diseases and susceptibility.

Mouse studies are uniquely suited to determine the effects of genetic variance on exposure to toxic chemicals, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin). Early studies revealed unique protein interactions with dioxin within mouse hepatocytes that are not observed in primate or human subjects and this suggested that mouse models would not be appropriate models for humans(Kimbrough). However, population-based mouse studies later took advantage of the diverse murine backgrounds to understand toxic responses of the genetically diverse human population as a whole. Those studies, such as response variations of the genotoxic components of murine benzene exposure or variations in the metabolism of butadiene, proved the relevance and benefits of using mouse populations in exposure studies that assess toxic environmental compounds(French et al., 2015; Hartman, 2017) .

Mouse studies often encompass a single strain when analyzing human outcomes associated with toxicity and disease outcomes of chemical compounds. It is emphasized that use of multiple mouse strains yields greater benefits when attempting to translate results into human relevance(Mosedale, 2018; van der Schalie et al., 1999). However, due to budget and time constraints, research continues to use single-strain mouse outcomes with those of the general human population. This undoubtedly limits the translational robustness of these models to the human population.

Due to factors such as age, development, and genetic background, humans vary in their susceptibility to detrimental outcomes of dioxin exposure(Beck, Dross, & Mathar, 1994; DeVito, Birnbaum, Farland, & Gasiewicz, 1995; Orban, Stanley, Schwemberger, & Remmers, 1994; Ryan, Gasiewicz, & Brown, 1990). We utilize the animal model in order to better understand the mechanistic actions of dioxins and identify human biomarkers of suceptibility(Portier, 2001). With this approach, we improve upon previous science-based risk assessment models.

I.2 Risk Assessment of TCDD and Related Compounds

I.2.1 Introduction

Dioxin and dioxin-like compounds are potent members of the polychlorinated dibenzo-p-dioxin (PCDD) family of compounds. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the most potent member of the PCDD family. It is a highly toxic compound whose cellular effects are mediated by aryl hydrocarbon receptor (AHR)(Poland, Glover, & Kende, 1976). Upon activation, AHR upregulates enzymes that elicit a spectrum of downstream toxic responses(Bohonowych & Denison, 2007; Sorg, 2014).

Chemists first discovered PCDD compounds throughout our ecosystem and identified them as byproducts of the synthesis of organochloride compounds(S. H. Safe, 1995). Their resistance to environmental degradation allows them to persist for long periods throughout the atmosphere, water, and soil(Hay, 1981; S. Safe, 1990). These ubiquitous environmental compounds cause a wide range of detrimental effects on human health and the ecosystem(Grassman et al., 1998). In the environment these compounds are

found as complex mixtures with congeners and other halogenated compounds, such as PCBs and dibenzofurans(Dickson & Buzik, 1993). The variation in toxicity of dioxin isomers, combined with the complexity of mixtures with related compounds, make assessing risks from exposure to these compounds very difficult(Barnes, 1991; Dickson & Buzik, 1993).

I.2.2 Origins and Properties of TCDD

PCDDs are commonly referred to as “dioxins,” signifying their overall structure consisting of two benzenes joined by two oxygen molecules. Dioxins consists of 75 different congener molecules with different substitution of the chlorine atoms on the two aromatic rings(Dickson & Buzik, 1993). PCDDs are highly lipophilic with lipid solubility increasing with the number of chlorine substituents and the hydrophobicity of these compounds allows them to bioaccumulate in adipose tissue. Turkish studies revealed the mean WHO-TEQ (World Health Organization toxic equivalents) human concentration of PCDDs of the general population to be around 9 pg/g fat(Cok et al., 2007). The half-life of PCDDs in humans varies due to differences in body composition, in which dioxin persists longer in individuals with a higher fat to lean ratio (Table 1-1) (Byard, 1987; Orban et al., 1994; Schecter, Birnbaum, Ryan, & Constable, 2006).

Analyte	Entire Nation 100[a]	Census Region				Age			Race		Sex	
		NE 22	NC 26	S 33	W 19	0–14 23	15–44 46	45 + 31	Caucasian 83	Non-Caucasian 17	Male 49	Female 51
PCDDs												
2,3,7,8-TCDD, pg/g	5.38	6.02	5.72	5.19	4.54	1.98	4.37	9.40	4.62	9.15	4.22	6.48
(RSE, %)	(6)	(10)	(11)	(11)	(16)	(41)	(12)	(4)	(14)	(29)	(11)	(8)
1,2,3,7,8-PeCDD, pg/g	10.7	11.3	10.8	10.4	10.3	3.30	9.33	18.2	10.6	11.4	10.8	10.6
(RSE, %)	(4)	(8)	(7)	(8)	(12)	(22)	(7)	(4)	(7)	(23)	(6)	(7)
1,2,3,4,7,8/ 1,2,3,6,7,8-HxCDD, pg/g	75.1	75.5	82.8	66.9	78.7	23.4	70.9	120	71.7	92.0	73.7	76.5
(RSE, %)	(4)	(8)	(6)	(7)	(8)	(23)	(6)	(3)	(7)	(19)	(6)	(5)
1,2,3,7,8,9-HxCDD, pg/g	11.7	10.7	12.7	11.5	11.7	6.13	10.8	17.1	11.5	12.3	11.0	12.3
(RSE, %)	(4)	(9)	(7)	(7)	(10)	(18)	(7)	(4)	(8)	(28)	(7)	(6)
1,2,3,4,6,7,8-HpCDD, pg/g	110	120	115	105	103	45.7	99.8	174	108	123	104	117
(RSE, %)	(3)	(7)	(6)	(6)	(9)	(11)	(5)	(3)	(5)	(14)	(5)	(4)
1,2,3,4,6,7,8,9-OCDD, pg/g	724	765	759	738	608	215	692	1150	718	755	676	771
(RSE, %)	(4)	(7)	(6)	(6)	(15)	(17)	(7)	(5)	(5)	(15)	(7)	(5)
PCDFs												
2,3,7,8-TCDF, pg/g	1.88	1.85	2.00	1.53	2.33	1.97	1.45	2.45	1.81	2.21	1.84	1.91
(RSE, %)	(7)	(14)	(14)	(15)	(14)	(11)	(15)	(7)	(12)	(33)	(11)	(11)
2,3,4,7,8-PeCDF, pg/g	9.70	13.7	10.5	9.51	4.49	1.87	8.00	18.0	9.25	11.9	8.70	10.7
(RSE, %)	(8)	(13)	(13)	(15)	(47)	(100)	(15)	(6)	(16)	(44)	(13)	(12)
1,2,3,6,7,8-HxCDF, pg/g	5.78	7.15	5.57	5.80	4.49	1.80	4.59	10.5	5.60	6.68	5.00	6.52
(RSE, %)	(13)	(27)	(24)	(25)	(43)	(83)	(26)	(13)	(25)	(73)	(24)	(19)
I-TEQ[b]	27.9	31.1	29.7	26.6	24.4	9.68	24.6	46.5	26.5	35.2	26.1	29.9

Note. For list of abbreviations, see note to Table 2.
[a]Population percentage based on 1980 US census.
[b]International Toxicity Equivalent (I-TEQ) includes an adjustment of 1.1 based on average composite concentrations of seven additional PCDFs.

Table 1-1. Estimated Average Concentrations (pg/g) With Relative Standard Errors (RSEs,%) For Selected PCDDs And PCDFs From FY 1987 National Human Adipose Tissue Survey Composite Samples (Reprinted from Orban et al., 1994)[*]. Examples of variations in human PCDD congener levels in adipose tissue.

TCDD is substituted with chlorines at positions 2, 3, 7, and 8; since this compound does not contain two adjacent unsubstituted carbon atoms the rate of metabolism is low and bioaccumulation in fatty tissue is high(Dickson & Buzik, 1993). TCDD is not directly used for industrial purposes but is a contaminant produced during synthesis of commercial chemical products, residential wood burning and forest fires, and these emissions lead to accumulation of TCDD and related compounds in the food chain(Czuczwa & Hites, 1984; Dickson & Buzik, 1993).

[*] Reprinted from "Dioxins and dibenzofurans in adipose tissue of the general US population and selected subpopulations" by J.E. Orban, J.S. Stanley, J.G. Schwemberger, and J.C. Remmers, 1994. *American Journal of Public Health*, Volume 84, Issue 3, Pages 439-445. Copyright 1994 by the American Journal of Public Health.

I.2.3 AhR: Ligands and their Structural Diversity

Early mouse exposure studies uncovered the mechanisms by which adaptive immune response minimized toxicity to ubiquitous environmental compounds. It was initially discovered that there was an increased expression of hepatic aryl hydrocarbon hydroxylase (*Ahh*) activity upon exposure to a number of toxic compounds, including benzo[a]pyrene (BaP) and 3-methylchlolanthrene (3-MC). This induction response was associated with enhanced cytochrome P450 (CYP)-dependent microsomal monooxygenase activity which, in turn, enhanced metabolism of BaP and related compounds(Schmidt & Bradfield, 1996). It was also reported that induction of Ahh activity was mouse strain-dependent and certain strains were designated as “responsive” and others as “non-responsive”. The discovery of dioxin’s high affinity for AHR later eliminated the idea of an “unresponsive strain,” as it induced a milder, yet toxic response(Poland & Glover, 1974),(Poland & Knutson, 1982b). Manipulation of the dominant responsive allele, Ahr^b, and the recessive “unresponsive” allele, Ahr^d, of inbred mouse strains led to the mechanistic model by which the *Ahr* encoded receptor proteins are essential to downstream induction of xenobiotic metabolizing enzymes, called cytochrome P450 enzymes (Cyp450s)(Ema et al., 1994; Poland et al., 1976; Poland & Knutson, 1982b). It was later discovered that the underlying polymorphism for mouse *Ahr* responsiveness involved a missense mutation (alanine to valine) at position 375 in Ahr^d allele (381 in human *AHR*) resulted in reduced affinity for dioxin(Ema et al., 1994).

AHR is a transcription factor responsible for mediating many biological processes and xenobiotic metabolism. It belongs to the basic-helix-loop-helix PAS superfamily that

mediates the expression of drug/xenobiotic metabolizing enzymes (DME/XME). Most of its toxic downstream effects, including tumor promotion, lymphoid involution, and porphyria, are activated upon binding halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs)(Poland & Knutson, 1982a; Schmidt & Bradfield, 1996). Previous researchers have consistently shown HAH ligands to yield the highest AHR-dependent toxic responses, and this is due in part to the persistent occupation of the receptor by these ligands. Many other compounds, including health-promoting flavonoids, pharmaceuticals, and microbial metabolites activate AhR-mediated responses, but not the toxic responses(Bock Karl & Köhle, 2009; Budinsky et al., 2014).

Within the cell, the inactive form AHR exists as a protein complex consisting of heat shock protein 90 (HSP 90), and other proteins(Sorg, 2014). Once activated upon ligand binding, AHR forms another protein complex with aryl hydrocarbon receptor nuclear transport (ARNT) protein. This complex then translocates into the nucleus, where it stimulates transcription of downstream toxic molecules, by binding cis-acting dioxin or xenobiotic response elements (DRE/XRE) in target gene promoters.

Through ligand activation, AHR functions within the metabolic, reproductive, and immune systems. Many AHR ligands activate their own metabolism and excretion upon binding, but some compounds can lead to the formation of DNA and protein adducts(Josyula et al., 2000; Nerurkar et al., 1996; Xu, Li, & Kong, 2005). Those ligands commonly include Halogenated aromatic hydrocarbons (HAHs) polyhalogenated aromatic hydrocarbons (PAHs), dioxins, indoles, and tryptophan derivatives(F. B, 2000; Bohonowych & Denison,

2007; Budinsky et al., 2014). HAHs and PAHs induce CYPs once bound to AhR, but HAHs are not readily metabolized and are mostly classified as toxic AhR ligands. PAHs are readily metabolized and are known to form active metabolites. These metabolites can interact with DNA, forming adducts, that may result in downstream effects, such as initiation of cancer. Other AhR ligands (i.e. flavonoids, some pharmaceuticals, synthetic metabolites, and tryptophan metabolites) induce CYPs on a tissue-specific basis. These ligands, unlike HAHs or PAHs, may or induce or modulate biological pathways that are not necessarily toxic, but often beneficial. For example, the pharmaceutical drug, omeprazole, follows a non-genotoxic Ahr-dependent pathway that does not allow nuclear translocation of AhR. Through the activation of Jun-N-terminal kinase (JNK) and other downstream genes have shown to be beneficial in inhibiting cell migration and invasion of Panc1 in pancreatic cancer when using omeprazole(Jin, 2018).

I.2.4 AhR: Genomic Pathways Include CYP Inductions

When bound to HSP90, AHR is inactive and unable to bind ARNT. Within the cytosol several chaperone proteins bind and maintain the inactive form of AHR(Sorg, 2014). This latent AHR complex incudes HSP90, Hepatitis-B virus X-associated protein 2 (XAP2), and prostaglandin E synthase 3 (p23)(Schmidt & Bradfield, 1996). XAP2 protects the inactive AHR from ubiquitination and degradation; and p23 is involved in the maturation, stabilization, and translocation of the AHR complex. These proteins are necessary for normal functioning of AHR activity, but much of the underlying mechanisms of p23 and XAP are still unknown(Schmidt & Bradfield, 1996; Sorg, 2014; Stockinger, Di Meglio, Gialitakis, & Duarte, 2014).

HSP90 and ARNT both exclusively interact with the bHLH and PAS domains of AHR. An AHR ligand, such as TCDD, enters the cell and binds AHR. The bound AHR is activated and releases its latent chaperone protein complex (Figure 1-3). ARNT then heterodimerizes with AHR and may stimulate the release of HSP90 and other proteins. The activated AHR-ARNT complex translocates into the nucleus and interacts with DNA, by binding to xenobiotic/drug response elements (XRE or DRE); which are contained within the promoter region of target genes(Schmidt & Bradfield, 1996). This interaction upregulates the transcription of Phase I and Phase II metabolizing enzymes (i.e. cytochrome P450 oxidative enzymes and UDP-glucuronosyltransferases, respectively). AHR signaling is subsequently down-regulated by the aryl hydrocarbon repressor protein (AHRR) and by ligand-induced proteasome-dependent degradation of the receptor(Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Nebert Dw Fau - Robinson, Robinson Jr Fau - Niwa, Niwa A Fau - Kumaki, Kumaki K Fau - Poland, & Poland, 1975).

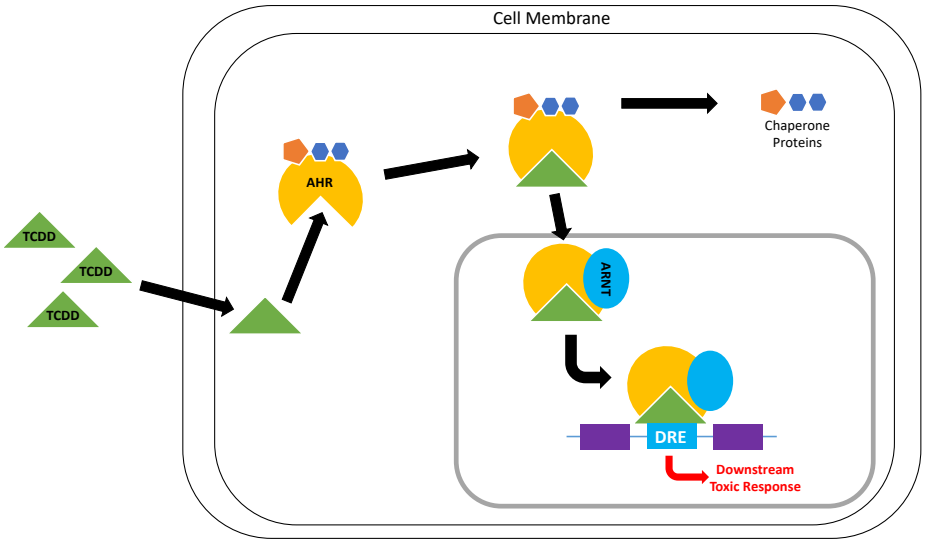


Figure 1-2. Dioxin Mode Of Action.

Cytochrome P450s (CYP) are AHR-mediated microsomal hemoproteins responsible for the activation and detoxification of many drugs and toxic compounds. TCDD-induced CYP1 enzymes commonly include CYP1A1, CYP1B1, and CYP1A2. The level of activation depends on the AHR allele and ligand bound.

I.2.5 AhR: Non-Genomic Pathways

The AHR also mediates effects independent of gene activation, and this has also been observed for many other intercellular receptors including the estrogen receptor and other nuclear receptors. These effects can be explained through AhR-ligand-dependent activation of a non-genomic pathway that do not require ANRT(Dong & Matsumura, 2008). For example, the rapid (~30 min.) increase in intracellular Ca^{2+} after dioxin exposure cannot be explained by genomic pathways, but through signal transduction activity of AHR(Nebert, Puga, & Vasiliou, 1993; Puga, Nebert, & Carrier, 1992). Another study reported immediate activation of ERK1 and ERK2 (within ~15 mins. of dioxin exposure) in human mammary epithelial cells and this was not accompanied by CYP1A1 induction(Park, Mazina, Kitagawa, Wong, & Matsumura, 2004). Researchers speculated that activated AHR alone specifically induces inflammatory actions of dioxin compounds(Matsumura, 2009).

I.3 Adverse Health Impacts of TCDD

I.3.1 Human Responses

I.3.1.1 Accidental/Industrial Exposures

Reports of acute high human exposures to TCDD have primarily occurred in chemical manufacturing and medical incineration plants(Calvert, Hornung, Sweeney, Fingerhut, & Halperin, 1992). Industrial incidents, like those seen in Seveso (Italy) and Nitro (West Virginia), and later through wartime exposures, laid the foundation for understanding the toxic effects of TCDD and related compounds. One of the earliest high dose industrial exposures, to TCDD were observed in a Monsanto Chemical Plant in 1949 and there were reports of chloracne, weight loss and fatigue in the exposed workers. There has also been concern regarding dioxin exposures that occurred during the Vietnam War (1962 – 1967). In 1976, a historical dioxin exposure occurred in Seveso, Italy, where a cloud of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was released into the environment during the production of 2,4,5-trichlorophenol(CDC, 1988). Much of the cloud debris settled into nearby soil and wildlife (birds, rabbits, chickens, etc.) many of which were found dead. Preliminary human reports from this incidence indicated ingestion or dermal exposure led to chloracne (i.e. dermal lesions)(CDC, 1988; Kimbrough). According to the incident, when a chemical plant exploded, even those with acute exposures to dioxin developed symptoms(Assennato, Cervino, Emmett, Longo, & Merlo, 1989). Similar animal exposure models administered lethal doses of TCDD exhibited wasting syndrome and weight loss several weeks post-exposure(Moore, 1978).

I.3.1.2 Agent Orange

The massive use of the defoliant Agent Orange during this war involved the largest amount of herbicide ever used within a country. US Military forces used this substance to defoliate in order to reveal enemy troops and destroy enemy crops. Veterans who were in contact with Agent Orange through handling, spraying and from environmental exposure exhibit increased prevalence of disorders in the thyroid gland, pituitary tumors, neurologic disorders, dermatitis (including chloracne) and liver damage, in both military personal and nearby residents(Assennato et al., 1989; Calvert et al., 1992; Tuyet-Hanh et al., 2015; Yi, Hong, Ohrr, & Yi, 2014). Many years after the war, Vietnam veterans had levels as high as of TCDD in adipose tissue(DeVito et al., 1995; Ryan et al., 1990). It was found that 2,3,7,8-tetrachloro-p-dioxin (TCDD) was found as a byproduct in the synthesis of 2,4,5-trichlorophenoxyacetic acid, the active ingredient of Agent Orange. The aftermath of Seveso and Vietnam exposures indicated that humans can tolerate around up to 50,000ppt (ng/kg) of TCDD concentrated in lipid stores without exhibiting major toxic side effects, excluding chloracne(Kimbrough).

I.3.1.3 Environmental Exposure

Trace amounts of dioxin existed in the environment prior to the industrial revolution due to “natural” combustion processes (i.e. fossil fuels), however levels greatly increased with increased industrialization and chemical manufacturing. These emissions were complex mixtures of polychlorinated dibenzo-p-dioxins and dibenzofurans(Czuczwa & Hites, 1984; Nagao, Golor, Hagenmaier, & Neubert, 1993). They are found in varying amounts in air,

water, wildlife and plants. The levels of human exposure can be variable based, with people living in more industrialized countries having higher levels. The human population has a general background level between 1-2 ppt TCDD in serum lipids and humans acutely exposed through consumption of fatty foods, such as fish, milk and meat; and exposure to relatively small amounts through air and water (Kimbrough, 1991; Tuyet-Hanh et al., 2015).

Dioxin's high affinity for lipids results in accumulation in adipocytes and leads to decreases in cellular metabolism(Orban et al., 1994; Ryan et al., 1990). Many effects of TCDD exposure resemble those of thyroid dysfunction, including weight loss, changes in hormone levels, and dermal anomalies, and altered immune fuction(S. Safe, 1990; S. H. Safe, 1995). Hepatic hypertrophy and hyperplasia is commonly due to prolonged AHR activation in most species and cytokine production is likely the cause of hepatotoxicity after prolong dioxin exposure in mice(Bock Karl & Köhle, 2009).

In 1997, IARC classified TCDD as a Group 1 carcinogen, deeming it harmful to humans. The validity of this assessment has been long debated since it is primarily based on AHR mechanisms of carcinogenesis. Dioxin-induced carcinogenesis is thought to be through a pathway that upregulates *Cyp1* enzymes, which correlates to the onset of tumor initiation and enhances promotion in cells that have already undergone initiation(Nebert, Dalton, Okey, & Gonzalez, 2004; Nebert et al., 1993). Upon substrate oxidation, CYP1 enzymes form reactive intermediates that result in downstream DNA damage, adduct formation, and generation of oxidative stress(Cole, Trichopoulos, Pastides, Starr, &

Mandel, 2003). Rodent hepatotoxicity studies revealed that dioxin's downstream products inhibit apoptosis and causes tumor promotion(Luebeck, Buchmann, Stinchcombe, Moolgavkar, & Schwarz, 2000; Pitot, Goldsworthy, Campbell, & Poland, 1980; Stinchcombe, Buchmann, Bock, & Schwarz, 1995). Genetic variation within the *Ahr* allele can also enhance dioxin-induced tumor promotion and progression, due to increased alterations in gene expression(Pitot et al., 1980).

I.3.2 Toxicokinetic Properties

TCDD is highly toxic to some animal and human species and has a wide inter- and intraspecies variation in response (Table 1-2). Over 1000-fold sensitivity differences have been reported in different rat strains that reveal these intraspecies difference in response in TCDD(Viluksela et al., 1996). Humans are acutely exposed to dioxin through toxic environmental mixtures. As the use of combustions processes increased in incineration and chemical production plants, so did the relevant background levels found in human tissues. The lipophilicity and stability of dioxin accounts for its biomagnification from reservoirs and sediment up the aquatic food chain into mammalian animals and humans.

The human lifetime body and tissue (i.e. muscle, adipose, etc.) burdens of dioxin result from ingestion of contaminated foods, and in rare occasions through industrial accidents (Figure 1-3). Dioxin congeners are the major PCDDs and PCDFs found in fatty foods consumed by humans, even though a wide array of similar congeners is released in the environment. High dose exposures occur mostly through industrial accidents or long-term exposure to toxic chemical substances that contain dioxin congeners (such as chemical

plant workers with inadequate protection from harmful compounds). Upon exposure, toxic effects are not elicited until the dioxin interacts with its target receptors (i.e. AhR) at sufficient concentrations and lengths of time.

ESTIMATED BODY BURDENS OF 2,3,7,8-TCDD/2,3,7,8-TCDD EQUIVALENTS (TEQ) ASSOCIATED WITH BIOCHEMICAL/BIOLOGICAL EFFECTS IN EXPERIMENTAL ANIMALS AND HUMANS

Effect	Species	Dose and duration[a]	Estimated body burden[b]
Acute lethality	Guinea pig	2.0 μg/kg, single dose	2000 ng/kg
Aryl hydrocarbon hydroxylase (AHH) induction	Rat	2.0 ng/kg, single dose	2.0 ng/kg
Suppression of serum complement	B6C3F$_1$ mouse	10 ng/kg/day, 14 days (ip)	96 (140) ng/kg[c]
Altered T cell subsets	Rhesus monkey	0.12 ng/kg/day, 1 year	32 (44) ng/kg
Chloracne	HRS/J hairless mouse	0.1 μg/day, 3 days/week, 6 weeks (dermal)	22,500 (90,000) ng/kg[d]
	Rabbit	4 ng/day, 5 days/week, 4 weeks (dermal)	25 (32) ng/kg
	Rhesus monkey	34 μg 2,3,4,7,8-PnCDF/kg (iv)	17000 ng TEQ/kg[e]
	Rhesus monkey	1 μg/kg, single dose	1000 ng/kg[f]
Nausea, anorexia	Human	PCDFs, several months	2200 ng TEQ/kg
Chloracne	Human	PCDFs, several months	2000 to 3000 ng TEQ/kg
Reproductive effects, impaired conception, spontaneous abortion	Rat	10 ng/kg/day, 1 year	434 (3650) ng/kg
	Rhesus monkey	50 pg/kg/day (NOEL)	50 ng/kg body fat[g]
		5.3 months (steady state)	11 ng/kg body wt
Cancer	Rat	10 ng/kg/day, 2 years	435 (7300) ng/kg

Note. Table modified from Universities Associated for Research and Education in Pathology (1988).

[a] All doses are for 2,3,7,8-TCDD and are oral or dietary unless otherwise indicated.

[b] Values were calculated based on determined and estimated first-order elimination.

[c] Values in parentheses indicate the body burden if there were no metabolism and/or elimination of 2,3,7,8-TCDD.

[d] On the basis of data of Puhvel *et al.* (1982) and assuming the body weight of the mice to be 20 g and the first-order elimination half-life to be 11 days, i.e., the same as the C57Bl/6 mouse.

[e] On the basis of data of Brewster *et al.* (1988); 90% of the dose was eliminated from the blood within 6 min of dosing and the whole-body half-life was approximately 49 days. Since two out of three animals died 40 and 48 days after treatment, the value of 17,000 ng TEQ/kg may be high relative to a lower dose that causes chloracne in the absence of lethality.

[f] On the basis of data of McNulty (1985).

[g] On the basis of the calculated steady state no-observable-effect level of 50 pg/kg/day determined by Bowman *et al.* (1989) and assuming 5.6 kg body wt and 14% fat per animal.

Table 1-2. Estimated Body Burdens of 2,3,7,8-TCDD/2,3,7,8-TCDD Equivalents (TEQ) Associated With Biochemical/Biological Effects In Experimental Animals And Humans (Reprinted from Ryan et al., 1990)*. Interspecies differences in TCDD effects based on dose, duration and body burden.

Once inside the body, the half-life of dioxin and dioxin-like compounds is dependent on age and body fat content. The TCDD body burden of children is diluted faster than adults

*Reprinted from "Human Body Burden of Polychlorinated Dibenzofurans Associated with Toxicity Based on the Yusho and Yucheng Incidents" by J.J. Ryan, T.A. Gasieicz, and J.F. Brown, Jr. 1990. *Fundamental and Applied Toxicology*, Volume 15, Issue 4, Pages 722-731, Copyright 1990 by Elsevier Inc.

due rapid growth, with smaller children having half-lives of about 4 months and adults having half-lives ranging around 7-10 years after exposure(Charnley & Kimbrough, 2006). The rates of eliminations decrease significantly with increased body fat burdens(Michalek & Tripathi, 1999). TCDD is shown to be primarily excreted through feces, seen in guinea pigs, rats and humans, with fecal lipids being a primary determinant of TCDD elimination rates(Rappe, 1992; Wendling, Orth, & Poiger, 1990).

Fetal and infant metabolism of PCDDs is largely unknown but are proportionate to maternal concentrations(Kreuzer et al., 1997). These levels differ after birth depending on feeding method, with breast-fed infants having 0.38-4.1 ng/kg of TCDD and formula-fed infants having 0.16-0.76 kg/ng of TCDD in adipose tissue(Kreuzer et al., 1997). As infants transition into adolescence, breast-fed infants show higher levels of TCDD levels in adipose tissue, but do not exceed normal background levels in adulthood (ranging from 2-3 ng/kg in serum lipid)(Kreuzer et al., 1997). Until the age of 40 PCDD burdens have shown to range between 6-12 TEQ/kg serum lipid(Patterson, 2004).

At low dioxin exposures, body fat percentage influences toxicokinetics, but at high exposures dioxin induces its own elimination(Edmond C, 2005). Dioxin is assumed to be at equilibrium across human organs and tissues, with a dose-dependent elimination half-life of 5 to 10 years(Kreuzer et al., 1997). The rate of elimination significantly decreases as age and body fat percentage increases(Edmond C, 2005; Michalek & Tripathi, 1999). Household mineral oils and activated charcoal can also aid in gastrointestinal absorption

and elimination of dioxins and similar compounds, but much is unknown of any effective pharmacotherapy useful in decreasing the effects of exposure.

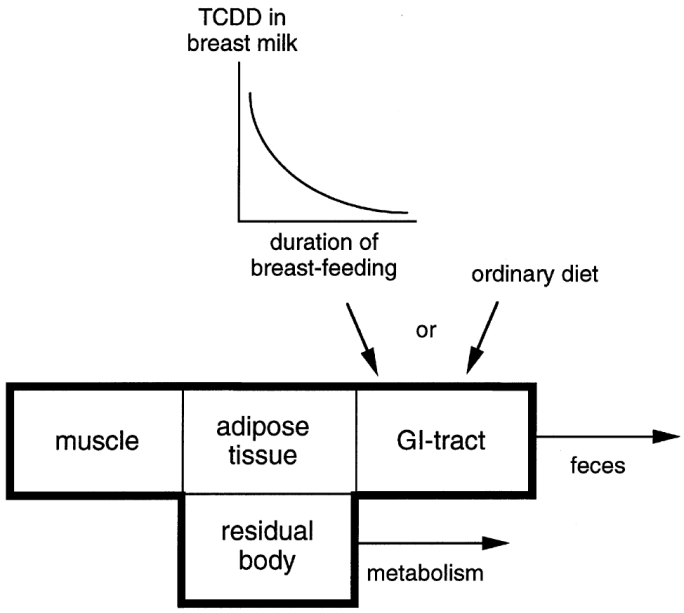


Figure 1-3. Toxicokinetic Model Based On Hysiological Parameters For The Description Of Lifetime Burden Of The Human Organism With TCDD (2,3,7,8-Tetrachlorodibenzo-P-Dioxin) Model Contains Physiological Parameters, Food Intake, TCDD Elimination By Metabolism, And Its Fecal Excretion As Age Dependent Variables(Reprinted from Kreuzer et al., 1997)*.

I.4 Effects of *In Utero* Exposure to TCDD

Although much is known about potential effects of dioxin in the environment, more is still to be uncovered about effects on mammalian embryonic systems or differential effects across individual backgrounds. The effect of genetics on response to embryonic exposure to dioxin is of importance because toxicant exposures are thought to cause several pregnancy complications. Dioxin action is mediated by AHR, which is present throughout female reproductive tissues and is involved in reproduction and development. If the AhR

* Reprinted from "2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and congeners in infants. A toxicokinetic model of human lifetime body burden by TCDD with special emphasis on its uptake by nutrition" by P.E. Kreuzer, G.A. Csanady, C. Baur, W. Kessler, O. Papke, H. Greim, J.G. Filser, 1997. *Archive of Toxicology*, Volume 71, Issue 6, Pages 383-400, Copyright 1997 by Springer-Verlag Berlin Heidelberg.

is inappropriately activated during pregnancy this may lead to developmental defects that are permanent and maybe persist through adulthood. These defects have been extensively characterized through mouse and zebrafish study and include skeletal malformations, restricted growth, neural defects, cardiovascular dysfunction and post-natal lethality(Carney, Prasch, Heideman, & Peterson, 2006; Couture, Abbott, & Birnbaum, 1990; Kimmel, 1996).

Consequently, dioxin exposure during pregnancy is of concern since it heightens the risk of fetal malformations, which can be incompatible with life, like the severe skeletal malformations of the skull and body seen during the aftermath of the Vietnam War. Although animal studies have shown that high exposure contributes to birth defects, human clinical data generated over the last few decades have only speculated that dioxin-induced toxicity as an underlying cause of some birth defects. Recently, combined human and animal data have shown correlations between increased dioxin exposure and the gradual increase in the occurrence of birth defects.

I.5 Applications of Genetically Diverse Mouse Models for Risk Assessment

The use of diverse mouse models in studying dioxin-induced birth defects will provide important new insights on the clinical relevancy of effects of this compound on the genetically diverse human population. The National Research Council (NRC) have addressed concerns of how interindividual variability of the human population effect epidemiological studies and the utility of using genetically diverse animal models to fill this gap in research(N. R. C. N. U. C. o. I. R. A. A. U. b. t. U. S. E. P. A. U. EPA), 2009).

Genetic variability is an important factor when assessing human exposures to environmental contaminants and can contribute to individual susceptibility or resistance to adverse exposure outcomes. Both intrinsic and extrinsic factors, such as age, genetic predisposition, nutrition, and exposure level, all determine the degree to which exposure response may vary(Zeise, 2013). Since risk assessment inherently involves individual variations of response, the current methodology must be redesigned to incorporate response variation found within the population to yield more accurate exposure assessments. When calculating exposure levels and effects in current risk assessment protocols uncertainties exist and are standardized through calculated response thresholds(U. S. E. P. A. U. EPA), 2014). These response thresholds do not accurately depict the response of a genetically diverse human population.

Due to the ethical limitations of human models in toxicant assessments, we use experimental models capable of characterizing relevant sensitivities and effects found within human populations. Classical risk assessment models utilize traditional rodent models in testing toxicity, but these rodent strains lack genetic diversity due to fixed genomes. These isolated gene pools allow researchers to easily reproduce data, while reducing variations in results. Historical reasoning for their use is the ability to compare data from commonly used rodent strains across a variety of chemical exposure studies (Harrill & McAllister, 2017). Recent studies have exposed that the major drawback of single-strain studies is that rodent strains of different genetic backgrounds may vary in sensitivity to the adverse health effects associated with the studied toxic compound. For example, studies evaluating the effects genetic variation has on hepatotoxic and

nephrotoxic outcomes of acetaminophen and trichlorethylene, have shown that some mouse strains have found to be resistant while others show increased sensitivity (Harrill AH, 2009; Yoo HS, 2015).

Population-based rodent studies reflect the genetic diversity found within the genetically diverse human population. It allows researchers to investigate the variety of toxic responses, including, reproductive and developmental toxicities, nephrotoxicity, and hepatotoxicity, at the population level. They are also key in identifying genetic risk factors that influence susceptibility and various disease states. In risk assessment, diverse mouse populations allow scientists to better understand how to quantitatively assess interindividual variability in the toxicokinetics of toxic chemical compounds. These populations highlight the incorporation of highly susceptible individuals into overall data analysis, which is otherwise nonexistent in single-strain studies. With this research, we hope to highlight the usefulness of population based rodent populations when predicting human hazards to toxic chemicals. Furthermore, this research will aid in the integration of the effects of genetics on differential responses into human risk assessments.

II PEANUT BUTTER AS AN ALTERNATIVE DOSE DELIVERY METHOD TO PREVENT OROGASTRIC GAVAGE-INDUCED STRESS IN MOUSE TERATOGENICITY STUDIES

II.1 Introduction

Orogastric gavage is a frequently used route of drug and toxicant administration for exposure studies. In this procedure, a gastric feeding needle is inserted through the oral cavity to the esophagus for direct delivery into the stomach(Balcombe, Barnard, & Sandusky, 2004). However, this method has been associated with stress and physiological changes, such as altered hormone levels, esophageal damage, weight loss, increased blood pressure and altered adrenal gland size and function in rodents(Arantes-Rodrigues et al., 2012; Balcombe et al., 2004; Brown, Dinger, & Levine, 2000; Dobrakovova & Jurcovicova, 1984; Murgas, Czako, & Dobrakovova, 1974). Procedure-induced stress can impede study measurements and results(Walker et al., 2012). This is of great concern when animal data are used in evaluating potential human health outcomes and exposure limits since stress can directly alter behavior, immune response, development, and reproduction(Brown et al., 2000; Nicolaides, Kyratzi, Lamprokostopoulou, Chrousos, & Charmandari, 2015; Stratakis, Gold, & Chrousos, 1995; Ziejewski et al., 2012).

An alternative oral administration through diet can be effective and less stressful on rodents(Overk, Borgia, & Mufson, 2011; Walker et al., 2012). Recent studies have shown reduced behavioral and physiological stress in mice when dose is administered through

diet via a treat method(Kapetanovic et al., 2006). An ideal cost-efficient alternative to dose administration would be to incorporate common foods, such as peanut butter or honey, as vehicle(Kuster et al., 2012). With the use of peanut butter (PB), mice are able to self-administer compounds under study. This delivery method is a more realistic model of exposure for assessing how animal exposure data translates into human exposure settings(de Meijer, Le, Meisel, & Puder, 2010; Kapetanovic et al., 2006).

Animal models are especially useful when evaluating teratogen exposures, as human data are almost always lacking. Evaluating the teratogenic effects of environmental compounds on humans during pregnancy is unethical, so the mouse model serves as a surrogate. However, due to increased susceptibility and physiological demands of pregnancy, studies requiring repetitive orogastric gavage can induce unacceptably high levels of procedure-associated stress that can complicate interpretation of experimental results(Balcombe et al., 2004).

To avoid added stress-induced physiological changes caused by orogastric gavage during pregnancy, we describe the use of peanut butter for self-administration in mice as a novel vehicle for dose administration. In our study, we utilize dioxin, an endocrine-disrupting compound, as a model compound for teratogen exposure. The primary hormones used to evaluate experimental stress are glucocorticoids, a class of steroid hormones which include cortisol/corticosterone(Brown et al., 2000; Dobrakovova & Jurcovicova, 1984; Gonzales et al., 2014). Prenatal influx of these hormones has shown to decrease fertility and impede development(Fawcett, S., Beckman, & Brent, 1996; Liu,

Dong, Wang, Cao, & Chen, 2014). We examined serum corticosterone levels, weight and reproductive endpoints to measure stress response of orogastric delivery versus self-administration.

Toxicologists have also become increasingly concerned with the genetic interactions of xenobiotics(Festing, 2001), which led to the growing field of toxicogenetics, in which human clinical relevance of animal data is improved through incorporation of genetic variability into animal studies. However, genetic heterogeneity of exposed human populations has rarely been replicated in animal pregnancy-associated exposure studies. Here we evaluated the impact of genetic heterogeneity on orogastric delivery during pregnancy using two different inbred mouse strains.

II.2 Materials and Methods

II.2.1 Animals

All animals were handled according to Texas A&M University's Institutional Animal Care and Use Committee (IACUC). Animals were obtained at 6-8 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Mice were housed as mating trios in ventilated cages on 12-hour light/dark cycles under constant temperature and humidity and were provided mouse chow (4% Fat Mouse Chow Diet (2025), Rodent Diet Tekland 8064, Envigo, Madison, WI) *ad libitum*. Peanut butter habituation also occurred during trio matings, in which mice were trained on a daily basis to consume peanut butter aliquots.

Females were checked daily between 6-10am for compilation plugs. Plug-positive mice were individually housed and assigned to the following groups until day 10.5 of gestation (D10.5): dry gavage (DG), oil gavage (OG), high-dose dioxin gavage (HG), control peanut butter (CPB), high-dose dioxin peanut butter (HPB), or a control do not disturb (DND) group (Table 2-1). The final dose for all mice was administered between 6-8am via oral gavage to ensure each mouse received a full dose prior to euthanasia between 11am-2pm.

Group	*Administration*	*Vehicle*	*Treatment*	*Dose Level (ng/kg/day TCDD)*	*Dose Volume*
Oil Gavage (OG)	Gavage	Olive Oil	Control	0 ng/kg/day	0.1 mL
High Dose Gavage (HG)	Gavage	Olive Oil	High Dose	100 ng/kg/day	0.1 mL
Control Peanut Butter (CPB)	Dietary	Peanut Butter	Control	0 ng/kg/day	3 g
High Dose Peanut Butter (HPB)	Dietary	Peanut Butter	High Dose	100 ng/kg/day	3 g
Do Not Disturb (DND)	None	None	Control (undisturbed)	0 ng/kg/day	None

Table 2-1. Experimental Design..

II.2.2 Dose Administration

II.2.2.1 Peanut Butter (PB) preparation

Dioxin doses were administered using 3g aliquots of PB based on initial weight of mice. Peanut butter was made by mixing 140g PB2® powder (Powdered Peanut Butter, Bell Plantation Inc., Tifton, GA) and 200 mL of ultrapure water (Milli-Q Advanced Water Purification System, MilliporeSigma, Darmstadt, Germany). Water was warmed and stirred on a hot plate until it reaching 40 ˚- 45 ˚C in a 500mL beaker. This recipe was repeated approximately 20 times to ensure accuracy of dosing when creating the final

protocol with dioxin. The required amount of dioxin for each dose group was then added along with a pinch (<1g) of PB2® powder and mixed for 5 minutes before the heat was turned off. PB2® was stirred in increments of 40g, 50g, and 50g, respectively. After mixing the final 50g, the mixture was vigorously stirred to ensure dioxin was distributed evenly. All aliquots were stored at 4 ˚C for no longer than 2 weeks.

Once dioxin-laced PB protocols were finalized, samples were sent to the Dow Chemical Company to measure and confirm the accuracy of dioxin in each dose. PB was presented to mice in 35mm x 10mm cell cultures dishes for self-administration, but the use of polystyrene was a major concern because of the hydrophobic nature of dioxin. To test the amount of residual dioxin that bound to the plastic, dioxin-laced PB was stored in the dishes for the maximum 2 weeks, emptied, and the dishes sent to DOW. Insignificant trace amounts were found on each dish, indicating that the storage conditions did not result in dosage errors.

II.2.2.2 PB Administration

Mice assigned to PB dosing groups were trained by giving 3g of control PB per cage three times a week to ensure the entire PB dose was consumed. Once in trio-matings, mice assigned to the PB groups were then given 3g of control PB per cage daily.

PB aliquots were gently placed into disposable cages to avoid disruption and reduce the external stress of cage handling. Dioxin PB was measured according to the weight of

each mouse at the initial dose (D1). There were a total of six dosing groups categorized by different weight ranges (Table 2-2).

Dioxin was administered in 3g aliquots of PB on 35mm x 10mm cell cultures dishes. Mice were given a daily dose through PB aliquots until D10, when last dose was administered via orogastric gavage.

Group	*Weight (g)*	*Average Weight (g)*
1	12g	10
2	12.01 – 17g	15
3	17.01 – 22g	20
4	22.01 – 27g	25
5	27.01 – 32g	30
6	≥32.01g	35

Table 2-2. Dioxin Peanut Butter Dose Category.

II.2.3 Body Condition, Composition and Weights

Bodily conditions were observed daily for signs of illness and stress. We monitored for hunched spines, scrunched coats, and any other signs that seemed unhealthy or out of the ordinary. Maternal body weights were recorded at gestation day one (D1) (initial dose) and D10.5 (euthanasia). Body weight and composition were measured on D1 and D10.5. Body composition was performed using an EchoMRI-100H^{TM} Body Composition Analyzer (EchoMRI LLC, Houston TX). Both weights and body composition results were reported as mean$\pm$SEM (Figure 2-2).

II.2.4 Serum Corticosterone (CORT) Levels

Blood samples were collected from each mouse immediately after euthanasia on D10.5 via cardiac puncture. Serum was collected using micro tubes containing gel with clotting activator (SARSTEDT; Order No. 41.1378.005). CORT levels were then measured by ELISA kit according to manufacturer's protocol (Cayman Chemical - Product 501320, Ann Arbor, MI). The assay range for CORT was 8.2-5,000 pg/mL, with an R^2 coefficient $\geq$ 0.990.

II.2.5 Reproductive outcome analyses

II.2.5.1 Implantation sites

The implantation sites on D10.5 were visibly identified as circular gestational sacs (about 5mm) found within the uterus(Mu, Slevin, Qu, McCormick, & Adamson, 2008). The uterus was further dissected to assess embryonic viability of each implantation site. Resorption sites or any other abnormality present were recorded. Embryos were further isolated to assign approximate developmental stage using *The Atlas of Mouse Development Edition 1* (Kaufmann, M.H., Elsevier Academic Press 1992).

II.2.5.2 Ovarian fat analysis

After blood collection, both ovaries, including surrounding ovarian fat, were collected and weighed. Ovaries underwent additional analysis for anomalies and size differences. Tissues were flash-frozen and stored at -80°C for analyses.

II.2.6 Statistical Analysis

Statistical analyses were performed using JMP Pro 12 (Cary, NC) statistical software and graphs were generated with GraphPad Prism 8 (San Diego, CA) software. One-way analysis of variance (ANOVA) tests were conducted on corticosterone levels, change in body weight, change in percentage of fat and lean weights, total implantation sites, and ovarian fat weight. For pregnancy rate, the experimental units used were female mice pregnant at the time of euthanasia at D10.5 and data were tested for independence by the chi-squared test. The data collected from pregnant mice within PB and gavage dose groups was compared to undisturbed pregnant control mice (DND) within each strain. Data was then compared across PB and gavage dose groups. If F static showed significance, the Dunnet's t-test for pair-wise comparisons was used for each dosing group. Data was presented at group means$\pm$SEM. Results were considered statistically significant if $p<0.05$.

II.3 Results

II.3.1 Effect of orogastric gavage on pregnancy is dependent on genetic background

We recorded the number of plug-positive mice and pregnancies found at D10.5 following continuous daily dosing from copulation to euthanasia. As shown in Table 2-3, 129S1/SvlmJ mice have a lower rate of pregnancy than C57BL/6J mice across all doses. Both strains showed a decrease in the rate of pregnancy, but 129SvlmJ mice have shown to have a much larger decrease in pregnancies during oral gavage administration. Pregnant 129S1/SvlmJ mice administered oil gavage (OG) yielded no pregnancies at euthanasia (D10.5, n=6). The average difference in pregnancies between PB and gavage administration in this strain is much higher than those seen in C57BL/6J mice (Table 2-3). Our results also show that C57BL/6J mice have a small decrease in pregnancies with gavage administration compared to PB administration. Although there is a significant difference in the rate of pregnancy for 129S1/SvlmJ undisturbed mice (DND) to both administration methods, peanut butter administration yields higher rates of successful pregnancies than oral gavage. There were no pregnancies in 129S1/SvlmJ mice administered OG so this group was excluded from all figures. Overall, our results suggest that oral gavage negatively affects pregnancy rates.

Strain	DND			CPB			HPB			DG			OG			HG		
	n[a]	Preg.[b]	Rate(%)[c]	*n*[a]	Preg.[b]	Rate(%)[c]	*n*[a]	Preg.[b]	Rate(%)[c]	*n*[a]	Preg.[b]	Rate(%)[c]	*n*[a]	Preg.[b]	Rate(%)[c]	*n*[a]	Preg.[b]	Rate(%)[c]
129S1/SvlmJ	19	12	63*	34	14	41	14	5	36	12	2	17	6	0	0	12	2	17
C57BL/6J	18	14	78	24	17	71	16	12	75	16	12	75	11	4	36	13	7	54

[a] *n* represents the total number of copulations plugs (i.e. plug-positive mice).
[b] Total number of mice pregnant at euthanasia (gestation day 10.5).
[c] (Total number of pregnant mice at euthanasia)/(Total number of copulation plugs).
* p<0.05; Pregnancy independent of administration method.

Table 2-3. Total Plug-Positive Mice And Successful Pregnancies Of 129S1/Svlmj And C57BL/6J Mice. After trio matings were set-up, mice were plug checked every morning. Plugs signified mating occurred overnight and were treated as potential pregnancies.

II.3.2 Orogastric gavage increases serum CORT levels in 129S1/SvlmJ mice

By examining the number of implantation sites within the uteri at D10.5, we were able to show that stress induced by dose administration methods had no effect on mean implantations in both 129S1/SvlmJ and C57BL/6J mouse strains (Figure 2-1). However, we did observe a decreasing trend in mean implantation sites in 129S1/SvlmJ mice that were administered dioxin PB. When comparing this finding to the same dioxin gavage dose (HG), HPB has a larger negative effect on implantation in 129S1/SvlmJ mice. Therefore, this effect suggests that teratogens, such as dioxin, administered through diet (PB) possibly have an increased delivery of dose. Due to genetic variation, C57BL/6J and 129S1/SvlmJ mice have significantly different responses to administration methods.

To further investigate whether oral gavage or peanut butter administration affected baseline stress levels, we analyzed the maternal physiological response to dosing conditions by measuring serum corticosterone (CORT) levels at D10.5. Serum CORT levels have no significant differences across all dose administration groups compared to the control group in C57BL/6J mice. On the contrary, an adverse outcome was observed in 129S1/SvlmJ dose administration groups. Mice administered oral gavage (dry gavage and high dose oil gavage) had significant increases in CORT levels, whereas the use of PB has no effect on CORT levels.

During early morning administration, mice administered oral gavage were hunched and resistant when handled, further indicating gavage-induced stress. The lack of pregnancies in 129S1/SvlmJ mice from OG group suggests that gavage itself induces

stress and oil addition as vehicle has some detrimental effects on pregnancy outcome. HG administration in 129S1/SvlmJ has a very large significant difference in mean CORT levels, compared to the controls, which implies underlying interactions between dioxin and CORT.

II.3.3 Impacts of Oral gavage on Body Weight and Composition

Mean body weight at the initial dose was not significantly different from the mean body weight at euthanasia between groups that received PB or gavage in both 129S1/SvlmJ and C57BL/6J pregnant mice compared to the control groups (Figure 2-2A). Oppositely, 129S1/SvlmJ mice that received gavage administration and were not pregnant at D10.5 showed significant differences in mean body weight at initial dose and euthanasia compared to control group (Figure 2-2B). C57BL/6J non-pregnant mice showed weight loss although not statistically significant, with the sole exception of OG administration being significantly different from both the control and CPB administration groups (Figure 2-2B).

Since we were able to detect differences in weight depending on administration method, we further analyzed body composition in both strains to evaluate changes in fat or lean mass at euthanasia in pregnant mice. We observed a significant difference in fat body mass in 129S1/SvlmJ mice when administered high dose gavage (HG) between initial dose and euthanasia compared to controls (Figure 2-2C). We also detected a significant change in lean body mass between initial dose and euthanasia in 129S1/SvlmJ mice when administered HPB and DG (Figure 2-2D). Although no significant changes were

retrieved in overall mean fat and lean body mass across all administration groups in C57BL/6J pregnant mice (Figure 2-2C and 2-2D).

We looked into ovarian fat weight to understand genetic \background correlates with previous knowledge of dioxins interference with ovarian function and fertility. There was no significance in mean ovarian fat weight in all administration groups in 129S1/SvlmJ pregnant mice. On the contrary, C57BL/6J pregnant mice showed a significant increase in ovarian fat weight mean when administered CPB and HPB, respectively, compared to controls (Figure 2-3). The same effect was also detectable when administered HG compared to controls in C57BL/6J pregnant mice although less significant (Figure 2-3).

II.3.4 Discussion

Previous investigators have extensively focused on the post-natal effects of maternally imposed stress in mouse animal studies. These studies have reported that offspring show various stress-induced abnormalities in development, such as growth retardation, craniofacial defects and adverse behavioral patterns(Lee et al., 2008; Liu et al., 2014). However, few studies have focused on the effects of oral dose administration on pregnancy outcomes, such as implantation rates, when evaluating endocrine disruptive actions of compounds in pharmacological and toxicological studies.

Developmental and reproductive animal studies are often used to assess the effects of environmental contaminants on human pregnancies and embryogenesis. Oral gavage is often preferred administration in environmental animal exposure studies but has often

reported to impose stress and increase mortality rates(Stratakis & Chrousos, 1995). Although much data has been generated on gavage-induced stress, none of these studies address how these affects confound reproductive and developmental data generated from past pharmaceutical and toxicological exposure studies.

In this study, we showed strain-dependent effects of oral gavage administration compared to dietary administration. The use of pregnancy as an overall endpoint was difficult to quantify as it depends on whether the animals will mate. Pregnant females were used to assess the effects of route administration on fecundity rates, body compositions, weight changes, and hormonal responses. 129S1/SvlmJ mice had lower fecundity rates when administered oral gavage, making calculating statistical significance of results difficult. Alternatively, the fecundity rates of C57BL/6J mice had no significant differences when administered oral gavage compared to controls and pregnant mice administered peanut butter (PB).

The mouse model is very efficient in assessing environmental exposures to harmful toxicants, but these studies often use one strain to generate results. When assessing human risks to environmental exposures, the heterogeneity of the human population must also be considered in order to more accurately identify genomic biomarkers of exposure(Harrill, Ross, Gatti, Threadgill, & Rusyn, 2009). Unfortunately, the animal model is limited in its ability to translate into relevant clinical data. However, collecting data from a genetically diverse mouse population increases the genomic data available, which

therefore enhances our ability to uncover clinical relevancy(Festing, 2001; Harrill et al., 2009).

In our study, we addressed how genetic background plays a pivotal role in the stress response to administration route during pregnancy. In addition, we evaluated how these effects impact results generated from common environmental exposure studies, such as *in vivo* dioxin exposure. In order to mimic the heterogeneity found within the human population, the use of multiple mouse strains is necessary and more representative when translated into human data. As shown in our results, neither of the strains utilized showed similar data or trends. The overall response was significantly different between strains.

The effects of administration method on fecundity were shown to be strain-dependent. This effect was not noticeable in the mean differences in weight, between D1 and euthanasia, of pregnant mice in both strains. The effects of administration and dioxin exposure were shown to be more significant in the weights of non-pregnant mice (i.e. mice that had no uterine implantations at euthanasia) than pregnant mice compared to their controls. During pregnancy weight gain is expected; which yields mean weight changes in pregnant mice to be negligible. With these expected weight changes, we are unable to determine whether the pregnant state yields protection against the effects of administration route on weight compared to the non-pregnant state. We also found the results of non-pregnant exposed mice to be beneficial to future pharmaceutical and toxicological studies that did not evaluate reproduction and development.

129S1/SvlmJ non-pregnant mice administered oral gavage showed a significant weight loss. The reduction in weight was even more significant in both OG and HG administration when compared to DG. This implies that the olive oil vehicle, combined with the oral gavage needle, induces more stress determining weight loss in 129S1/SvlmJ mice. Therefore, we speculate that a less viscous vehicle could potentially reduce stress administration-induced in this particular strain. Mean weight changes in non-pregnant C57BL/6J mice administered OG were significantly different from the non-pregnant controls and CPB administration. In this strain, delivery method plays a pivotal role in accuracy of results and has the potential to confound data.

Dioxins are known to have an effect on female fertility. One aspect is by compromising ovarian function and altering endocrine hormone levels involved in fertility and reproduction(O et al., 2011). Lower ovarian weights and follicular phase disruption has been shown to correlate with acute dioxin exposure in rodents(BK et al., 2001). We measured the change in ovarian fat weight (which includes ovaries) to analyze how this knowledge correlates with genetic variability. Although there were no significant differences in 129S1/SvlmJ mice, opposite results than what is shown in previous research were shown in C57BL/6J mice. The increase in C57BL/6J ovarian weight, when administered the peanut treat method, could possibly be due to decreased stress of using the peanut butter method. On the other hand, the C57BL/6J high dose gavage group implies the lipophilicity of peanut butter and/or dioxin could be attributing to the increased weight or overall health of the ovaries.

We also measured CORT levels to evaluate stress levels. In response to external stressors, the anterior pituitary is stimulated to release corticotropin (ACTH). This stimulation causes downstream effects that results in the release of glucocorticoids (cortisol/corticosterone)(Nicolaides et al., 2015; Stratakis & Chrousos, 1995). Responses to these molecules have been implicated to disrupt homeostasis, which detrimentally affects the immune response, growth and development, and reproduction(Stratakis & Chrousos, 1995; Stratakis et al., 1995).

Proper function of the immune response and hypothalamic-pituitary-adrenal axis is crucial in maintaining homeostatic balance of normal pregnancies and fetal development(Mu et al., 2008; Stratakis & Chrousos, 1995). Animal stress response induces HPA axis function, which leads to increased production of glucocorticoids(Nicolaides et al., 2015; Stratakis & Chrousos, 1995). Hence, CORT, a glucocorticoid produced in mice, and cortisol (in humans) are typically used as hormonal indicators of stress. It has been shown that increased prenatal levels of corticosterone play a key role in the formation of cleft pallet in offspring, implantation rates, decreased birth weights, and pre-term deliveries (Fawcett et al., 1996; Liu et al., 2014; Ziejewski et al., 2012).

In our study, we obtained blood samples from animals at D10.5 via cardiac puncture at necropsy; we then measured serum corticosterone levels of each animal. C57BL/6J mice had no significant differences in CORT levels across all dosing groups. On the other hand, CORT responses in 129S1/SvlmJ were significantly different suggesting that genetic background plays a role in dose response. 129S1/SvlmJ mice administered daily gavage

all had significant increases in CORT levels. These levels increased from DG to HG administration compared to controls. The use of a gavage needle alone has shown to be the cause of increased stress in 129S1/SvlmJ mice compared to controls. Unfortunately, we are unable to determine whether adding the olive oil vehicle alone causes increased stress, due to fecundity rates OG administration. We speculate that the presence of the oil, either with or without the compound, has an even more negative effect on stress of 129S1SvlmJ mice administered gavage.

Our results, along with previous studies, establishes key principles in understanding how the mouse model is affected by stress and how these effects decrease accuracy of measured outcomes(de Meijer et al., 2010; Gonzales et al., 2014; Kapetanovic et al., 2006; Kuster et al., 2012). The use of peanut butter as an alternative method of oral administration demonstrates increased efficacy of drug exposure in sensitive mouse models, including environmental exposure studies(Walker et al., 2012; Wheeler, Eppolito, Smith, Huff, & Smith, 2007). Many toxicological and pharmacological studies use diet and oral gavage interchangeably as means of dose administration. Our study has shown that these two methods should not be assumed to yield identical results. Dietary administration is preferred, as it yields a reduced stressed response in some strains, but not so much in other strains.

II.4 Figures

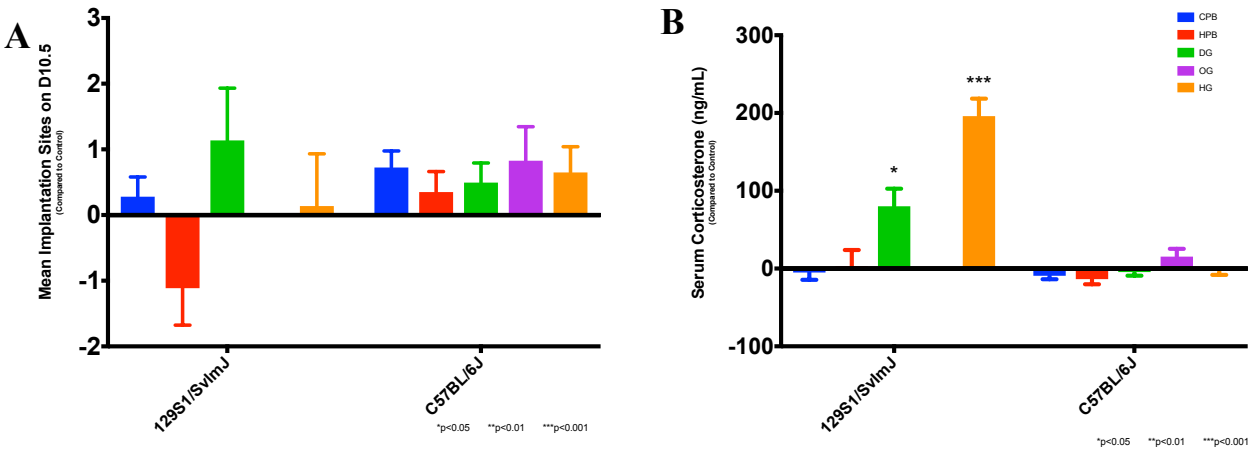


Figure 2-1. Mean Implantation Sites And Corticosterone Levels Of Pregnant Mice Compared To Controls (DND). (A) The number of implantation sites found at euthanasia was recorded for each administration group within both strains. Dose administration method had no effect on mean implantations found with uteri compared to controls within each strain. (B) Blood serum was also collected and serum corticosterone levels were measured via ELISA. Strain-dependent effects of administration methods on serum CORT levels compared to controls. Data expressed as mean values ± SEM (*p<0.05, **p<0.01, ***p<0.001, ANOVA).

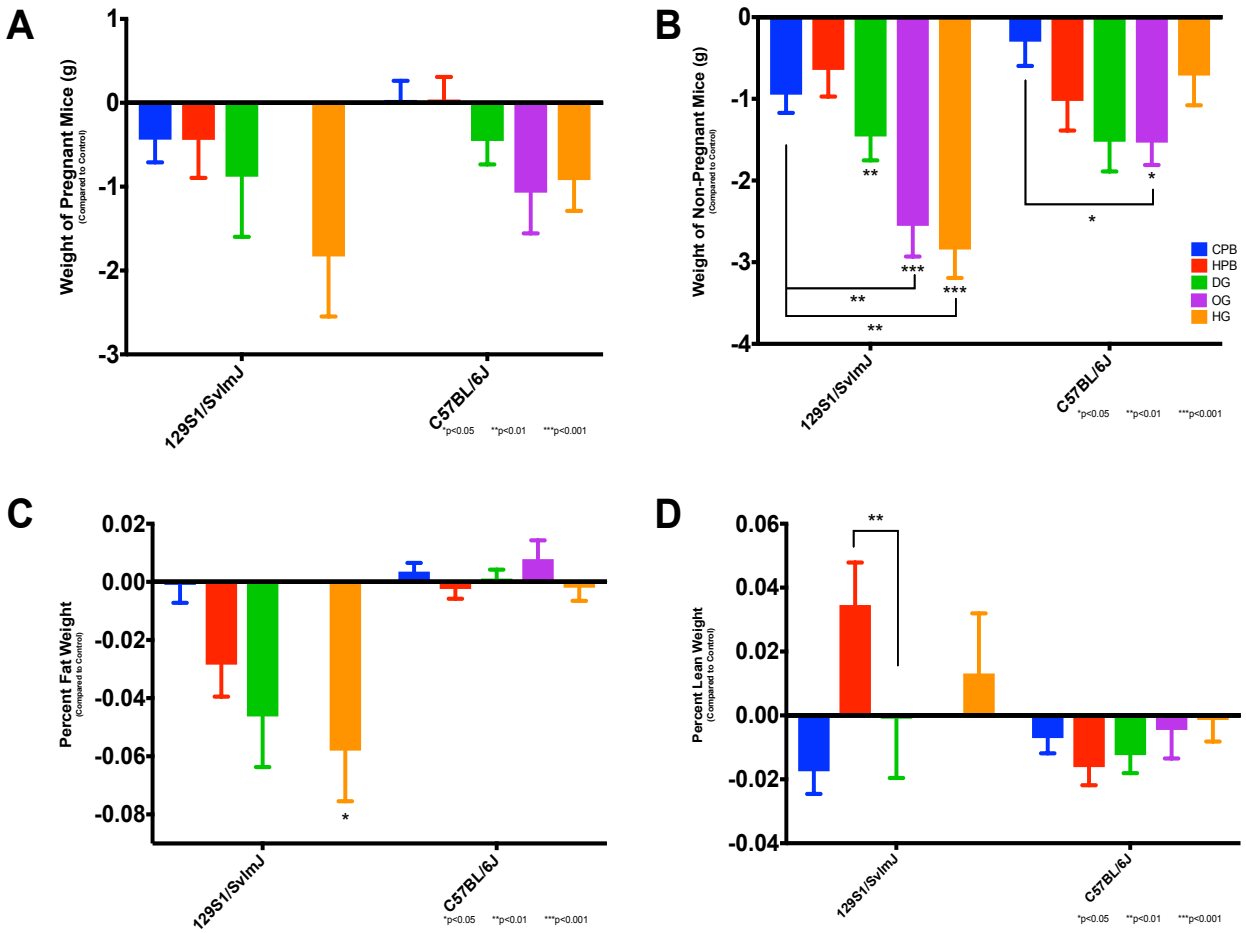


Figure 2-2. Percent Weight And Body Composition Changes. (A) Mean change in body weight (g) of pregnant mice between gestation day 1 and euthanasia compared to pregnant controls. (B) Mean change in body weight (g) of non-pregnant mice between gestation day 1 (D1) and euthanasia compared to non-pregnant controls. (C) Mean change in percentage of fat weight of pregnant mice compared to pregnant control mice. The change in weight is a measure of the difference between fat weight at D1 and D10.5. (D) Mean change in percentage of lean weight of pregnant mice compared to pregnant control mice. The change in weight is a measure of the difference between lean weight at D1 and D10.5.

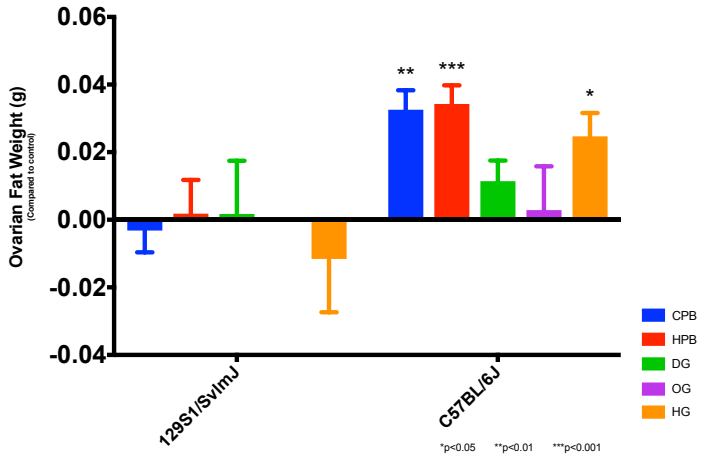


Figure 2-3. Ovarian Fat Weight In Pregnant Mice. Mean ovarian fat weight (g) in pregnant mice at euthanasia on gestation D10.5 compared to control groups in both 129S1/SvImJ and C57CL/6J strains.

III A POPULATION-BASED APPROACH TO ASSESS THE TERATOGENIC EFFECTS OF 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN EXPOSURE DURING PREGNANCY

III.1 Introduction

Environmental toxicants, such as dioxin, have become of increasing concern to pregnant women. Dioxin activity is mediated by the aryl hydrocarbon receptor (AHR), which is present throughout female reproductive tissues and is involved in reproduction and development. AHR is encoded by four different alleles in mice: Ahr^{b-1}, Ahr^{b-2}, Ahr^{b-3} and Ahr^{d}. The mouse strains having different alleles manifest different dose response relationships for TCDD toxicity. Mice that harbor Ahr^{d} allele are considered to be much "less responsive" or "nonresponsive" to dioxin exposure, and more genetically identical to the human *AHR* allele(Schmidt & Bradfield, 1996; Sorg, 2014; Thomas, Penn, Holden, Bradfield, & Rank, 2002). During pregnancy inappropriate AHR activation can lead to developmental defects that persist through adulthood(Sorg, 2014; Wang, Wang, Wu, Li, & Lu, 2013). Due to this sensitive developmental window, human studies are unsuitable for investigating dose-response relationships.

Ahr in murine models has been found to not only play a vital role in xenobiotic metabolism, but also in cellular regulation and developmental processes(Nebert et al., 1993; Sorg, 2014). AHR can contribute to altered gene expression and the onset of disease throughout development when its activity is deregulated. Potent environmental factors,

such as dioxin, have the ability to alter AHR activity and related developmental programs that result in the onset of diseases, including congenital heart defects. Consequently, dioxin exposure is a particular concern during pregnancy as it heightens the risk of fetal malformations that can be incompatible with life. Acute, high dose exposures to dioxin during the Agent Orange use in the Vietnam War and the chemical plant Explosions in Seveso, Italy have been linked to decreased growth, structural and functional malformations, and fetal death(Eskenazi et al., 2003; Schecter et al., 1995; Warner et al., 2007). Although these early studies have phenotypically showed the link between high exposure dioxin and birth defects, human accidental exposure data generated have only speculated dioxin-induced toxicity as the underlying cause.

Dioxin's widespread environmental distribution is a major health concern among pregnant women(Couture et al., 1990; Seo et al., 1995). Early murine embryo/fetal toxicity studies revealed hydronephrosis, cleft palate, thymic atrophy, fetal mortality, and decreased birth weight in response to dioxin exposure(Couture et al., 1990). Maternal data reveal early signs of toxicity correlating with decreased weight. Although the effects of dioxin have been investigated during pregnancy in mice, those studies have evaluated dioxin toxicity in a single-mouse strain(Abbott, Held, et al., 1999; Dragin, Dalton, Miller, Shertzer, & Nebert, 2006; Takagi, Matsui, Yamashita, Ohmori, & Yasuda, 2000). C57BL/6J mice are a commonly used mouse strain, but their genetic makeup does not represent the genetic diversity found within the general human population. Early exposure studies referred to a single mouse strain when analyzing exposure risks, and the resulting developmental

effects, such as cleft palate, became a great concern among pregnant mothers in high exposure areas.

Researchers later became more interested with the differences in inter-species responses, which showed significant differences in developmental responses(Dorne, 2004, 2010; Reichard et al., 2016). But this data is significant in assessing environmental exposure and tells us little on how these effects may vary between individuals of the same species. Genetic variation likely underlies differential susceptibility to adverse outcomes induced by dioxin exposure(Dorne, 2010). Therefore, the current study utilizes a genetic heterogeneous mouse panel to better model human exposure to dioxin.

III.2 Methods

III.2.1 Animals

Mouse strains were chosen from Mouse Phenome Diversity (MPD) Panel and were selected due to their favorable pathological and physiological comparisons to the level and types of response variations found in the human population (C57BL/6J, A/J, NOD/ShiLt, NZO/HiLt, and 129S1/SvImJ). Additional inbred strains were chosen to have significantly high fecundity rates (CBA/J, C3HeB/FeJ, DBA/1J, FVB/NJ, and BALB/cJ) (Figure 3-1). The MPD Panel strains were supplemented with strains from the Collaborative Cross and BXD recombinant inbred panels that also have high fecundity (CC019/TauUNC, CC041/TauUNC, BXD40/TyJ, BXD91/2RwwJ, and BXD100/RwwJ). Mice were provided water and standard mouse chow (Rodent Diet Tekland 8064, Envigo,

Madison, WI) *ad libitum*. Mice were set-up in trio matings (2 females and 1 male) and checked daily for copulation plugs to establish embryonic timing; noon on the day that plugs were detected was assigned embryonic day (E) 1. All procedures were approved by and performed according to Texas A&M University IACUC guidelines.

III.2.2 TCDD Preparation

Dioxin doses were administered using 3g aliquots of peanut butter (PB) based on initial weight of mice. Peanut butter was made by mixing 140g PB2® powder (Powdered Peanut Butter, Bell Plantation Inc., Tifton, GA) and 200 mL of ultrapure water (Milli-Q Advanced Water Purification System, MilliporeSigma, Darmstadt, Germany). Water was stirred and warmed up to 40 ˚- 45 ˚C on a hot plate in a 500mL beaker. This recipe was repeated approximately 20 times to ensure accuracy of dosing when creating the final protocol with dioxin. The required amount of dioxin for each dose group was then added along with a pinch (<1g) of PB2® powder and mixed for 5 minutes before the heat was turned off. PB2® was stirred in increments of 40g, 50g, and 50g, respectively. After mixing the final 50g, the mixture was vigorously stirred to ensure dioxin was distributed evenly. All aliquots were stored at 4 ˚C for no longer than 2 weeks.

III.2.3 TCDD Administration

At 3 weeks of age, mice were habituated to eat a defined amount of PB, the vehicle for dioxin oral delivery, on 35mm x 10mm cell cultures dishes. Mice were given 3g of PB per

cage 3 times a week to ensure the entire PB dose was delivered. At 4-6 weeks of age, mice were placed in trio-matings as described above and assigned to a dose group.

Dioxin was administered in 3g aliquots of PB. Mice were given their respective dose daily through PB aliquots until E10, when the final dose was administered via oral gavage (Figure 3-2). The final dose was given via oral gavage to ensure the complete dose was administered and in the digestive tract prior to euthanasia, which followed later the same day. We exposed a total of 14 strains of pregnant female mice to increasing doses of TCDD (0 control, 1, 100 ng/kg body weight/day) for a period of 10 days following mating (Figure 3-2). Each female in the trio-matings was checked daily for the presence of a copulation plug.

III.2.3.1 Sample Collection

Prior to initial (E1) and final (E10) doses, mice were weighed and body compositions were assessed using magnetic resonance imaging (MRI). At day E10.5 mice were anesthetized with 1.25% Avertin (tribromoethanol; 0.018ml/gram of body weight category) for blood collection, then euthanized by cervical dislocation for organ and tissue collection. The gross anatomy of the uterus was observed and recorded, along with the number of implantation sites. Each uterus was further dissected to assess tissue viability of each implantation site. The uterus was further dissected under a microscope to remove each deciduae. The uterus was then flash frozen for gene-expression analysis.

III.2.3.2 Implantation Site Examination

The implantation sites on E10.5 were visibly identified as circular gestational sacs (about 5mm) within the uterus(Mu et al., 2008). The number of viable embryos, and number of resorption sites were recorded. Embryos were further isolated to analyze developmental stage. Embryo implantation and resorption sites were determined with the use of *The Atlas of Mouse Development Edition 1* (Kaufmann, M.H., Elsevier Academic Press 1992). Developmental stages were determined by comparison to images provided in atlas. Half of the viable embryos recovered were stored in RNAlater (ThermoFisher Scientific) and the other half were fixed in formalin.

III.2.4 Statistical Analysis

Statistical analyses were performed using JMP Pro 12 (Cary, NC) statistical software and graphs were generated with GraphPad Prism 8 (San Diego, CA) software. One-way analysis of variance (ANOVA) tests were done on change in body weight, percentage of resorptions, and total implantation sites. If an F static showed significance, the Dunnet's t-test for pair-wise comparisons was used for each dosing group. Data were presented at group means $\pm$ SEM. Results were considered statistically significant if $p<0.05$.

III.3 Results

III.3.1 Effect of TCDD Exposure on Pregnancy Rates

We initially examined the effects of dioxin exposure on successful pregnancy (or implantation). Doses were chosen based on low dose region of the previous dioxin exposure paradigm. We chose the low dose end to ensure we were better able to identify individual strains with high susceptibility. In mice we found a variation in fertility/pregnancy rates in response to dioxin. Some strains showed no overt signs of being affected by exposure, compared to controls. There is a slight, but not significant, gradual decrease in pregnancy rate when comparing across controls to high dose groups in BXD100/RwwJ, 129S1/SvlmJ, and NZO/HiLtJ mouse strains (Table 3-1). But some mouse strains, including C57BL/6J, FVB/NJ, CC-19, and DBA/1J, have increasing trends in fertility when comparing controls to high dose groups. The effects of dioxin do not appear to be as significant on fertility at the low dose range.

III.3.2 Effect of TCDD Exposure on Implantation Sites

The result shown above suggested that dioxin exposure slightly decreases pregnancy rates in certain genetic backgrounds, so we examined its effects on the total number of implantation sites found within pregnant mice. Results were grouped by *Ahr* allelic differences of each strain. Our results show mice are grouped based on strain *Ahr* allelic background, to evaluate the differences in previous mouse AHR allele data and current results. There were few significant differences in mean implantations sites found within

the uteri if mouse strains with the B2 AHR allele and one D allele strain (Figure 3-4). BALB/cJ mice showed a significant increase in mean implantation sights at the high dose, but not at the low dose compared to the control group. In contrast, C3HeB/FeJ and A/J mice administered the low dose (1 ng/kg/day) had a significant increase in mean implantation sites at the low dose, but not the high dose compared to the controls. NOD/ShiLtJ mice showed a very significant decrease in mean implantations at the low dose, but not at the high dose compared to the controls. These results suggest that when the effects of dioxin are compared across multiple genetic backgrounds of the same species, AHR allelic differences seems to have little to no significance in interpreting results.

III.3.3 Effect of TCDD Exposure on Resorption Sites

To account for inter-strain differences in mean number of implantation sites, we determined the intra-strain differences in the percentage of resorption sites at each dose of dioxin. There were no significant differences in the mean percentage of resorptions found within implantation sites, except for the 129S1/SvlmJ (SV) mice (Figure 3-3). At the high dose (100 ng/kg/day TCDD), 129S1/SvlmJ mice had a significant increase in the percentage of resorptions. Some strains including, NZO/HiLtJ, NOD/ShiLt, BXD40/TyJ, C3HEB/FeJ, A/J, and BXD100/RwwJ, showed an increasing trend in the percent of resorptions when comparing control groups to high dose groups, but there were no significant differences in response.

III.3.4 Effect of TCDD on Mean Changes in Weight During Pregnancy

The previous results determined that endpoints related to pregnancy and fertility are strain-dependent, with some strains not experiencing certain effects to dioxin exposure. We then examined the change in maternal body weight between gestation days 1 (E1; copulation plug) and 10 (E10; euthanasia) to determine maternal effects of dioxin exposure during pregnancy. CBA/J mice showed a very significant decrease in weight at the high dose, but not the low dose compared to the controls. A/J mice dosed at the high dose had a significant increase in weight compared to the controls (Figure 3-5). On the other hand, BXD100/RwwJ mice given the low dose had a very significant decrease in weight compared to the controls.

III.4 Discussion

Previous teratogenic studies do not address genetics as a major player in responses to environmental exposures(Churchill et al., 2004; Dorne, 2004). We have studied the effects of acute dioxin exposures on pregnancy and genetic susceptibility. Our results help to expand upon the current knowledge of the impacts genetics has on individual susceptibility. Each endpoint investigated in our study showed variation, in which no one strain had the same trends or significant differences across more than one measured endpoint.

To incorporate genetic background into dose response studies, we utilized 14 different inbred mouse strains to model genetic diversity. These strains varied in their response to dioxin through their differences in AHR alleles. There is considerable data suggesting AHR's pivotal role in dioxin response, but comparable studies have not been assessed in multiple genetic backgrounds(Schmidt & Bradfield, 1996; Thomas et al., 2002). Mice have four variations of this allele: The B "responsive" allele (with 3 variations) and the D "non-responsive" allele(Harper, Golas, & Okey, 1991; Nebert et al., 2004; Wang et al., 2013). According to our data, the *Ahr* allelic status is not the primary factor mediating dioxin-induced pregnancy effects.

Pregnancy was assessed by various endpoints that are widely accepted as fertility and pregnancy markers. Several studies have also linked dioxin to infertility in both the male and female reproductive systems(Couture et al., 1990; Eskenazi et al., 2010). Pregnancy was initially assessed in this study with percentages of pregnancies, which is supported in multiple publications as an indicator of change in pregnancy rate or fertility(Aragon, Kopf, Campen, Huwe, & Walker, 2008; Couture et al., 1990; Eskenazi et al., 2010; H & R, 1981). We found that strains with genetic backgrounds comparable to C3HeB/FeJ are primarily unaffected by exposure to dioxin and dioxin-like compounds. Conversely, strains such as BXD100/RwwJ and NZO/HiLtJ experienced slightly higher detrimental effects of dioxin on pregnancy rate (or fertility) compared to control groups. Despite the obvious inter-strain differences in fertility, we found no significant differences in the relative rate of pregnancy at the low and high dose groups compared to the controls.

Implantation is consistently used as markers of successful pregnancies in various studies, but many do not further assess the implantation sites for embryonic viability(F. B, 2000; L. B et al., 2006; M et al., 2004). We then examined the percent of viable embryos found within each implantation site. We found variation in response that did not have similarities to other endpoints. Significant differences in resorption percentages were only found in 129S1/SvlmJ mice, which showed a significant increase at the high dose compared to the controls. This made it evident that 129S1/SvlmJ mice are more susceptible to increased resorptions (or decreased embryo viability). Other strains were not significantly affected by dioxin at this endpoint, but some showed to have more detrimental effects at different endpoints.

There are several reports evaluating the relevance of weight loss and dioxin exposure in both pregnancy and nonpregnancy related exposure studies(Couture et al., 1990; E, W, M, S, & C, 2014; Kimbrough; Schecter et al., 2006). Our weight related results have shown to be consistent with our previous data, as interstrain differences affect the variation in response to each endpoint a few strains made it evident that comparable backgrounds may have the same level of susceptibility to weight changes in response to dioxin exposure. Weight change could also possible be an indication of embryo viability or successful implantations.

Our results showed that individual response depends on both genetic background and the endpoint being measured. Each endpoint had different dynamics in response within and across each strain. Overall, these results have shown the influence of genetic

background on pregnancy-related consequence in a range of physiological and physical responses to dioxin response.

In conclusion, we mimicked human heterogeneity by using multiple genetically-diverse mouse strains in order to investigate the how genetic background contributes to variation in responses to dioxin. Our results showed that dioxin exposure resulted in variation in pregnancy implantation rates and resorptions, as well as maternal body weight, varied across mouse strains. demonstrated that population variability should be considered when determining dose response to toxicants found through the environment. This model is an effective tool in modeling susceptibility and adverse health effects in risk assessment. It is also important in assessing sensitive experimental model such as pregnancy. This information may be considered later when producing medical interventions or treatments for susceptible individuals living in areas where exposure to dioxin and dioxin-like compounds is prevalent. This data also prevalent when choosing the appropriate mouse strains for strain-specific functional experiments.

III.5 Figures and Tables

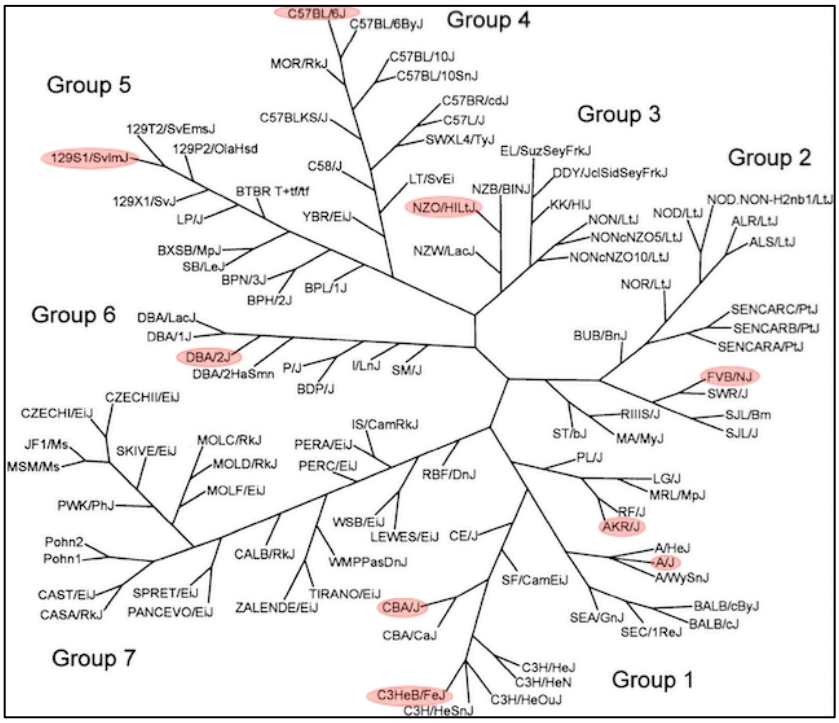


Figure 3-1. Inbred Mouse Family Tree Showing The Genetic Relatedness Of Some Of The Strains Used In This Study (Circled). (Adapted from Petkov, P. M., et al. 2004)*

* Adapted from "An Efficient SNP System for Mouse Genome Scanning and Elucidating Strain Relationships" by P.M. Petkov, et al, 2004. *Genome Research*, Volume 14, Pages 1806-1811, Copyright 2004 by Cold Spring Harbor Laboratory Press.

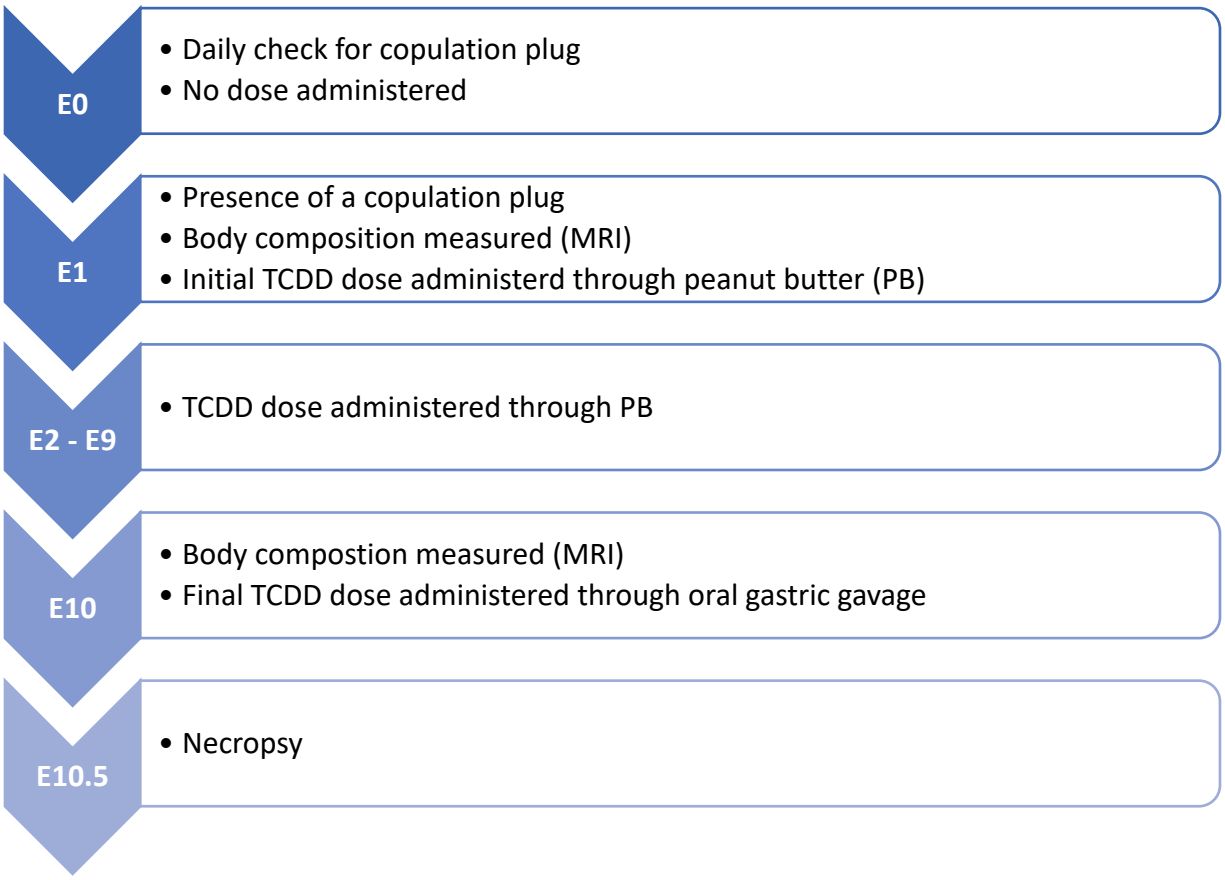


Figure 3-2. Experimental Timeline.

Strain	Control			Low Dose			High Dose		
	Positive Plugs	Pregnancies	Percent Pregnant	Positive Plugs	Pregnancies	Percent Pregnant	Positive Plugs	Pregnancies	Percent Pregnant
BXD100/RwwJ (B1)	8	8	1	10	8	0.8	10	7	0.7
C57BL/6J (B1)	16	11	0.69	10	10	1	16	12	0.75
A/J (B2)	16	13	0.81	12	8	0.67	12	9	0.75
BALB/cJ (B2)	17	9	0.53	15	7	0.47	11	6	0.55
C3HeB/FeJ (B2)	10	10	1	10	10	1	8	8	1
CBA/J (B2)	8	8	1	7	7	1	12	11	0.96
FVB/NJ (B2)	18	12	0.67	12	9	0.75	9	8	0.89
129S1/SvImJ (D)	14	7	0.5	11	4	0.36	14	5	0.36
BXD40/TyJ (D)	7	6	0.86	10	9	0.9	9	7	0.78
CC-19 (D)	10	7	0.7	5	4	0.8	7	6	0.86
DBA/1J (D)	13	10	0.77	7	6	0.86	12	10	0.83
NOD/ShiLtJ (D)	8	8	1	9	9	1	9	8	0.89
NZO/HiLtJ (D)	8	6	0.75	8	5	0.63	12	7	0.58

Table 3-1. Total Positive Plugs And Pregnancies. Total plug-positive mice and successful pregnancies of all mouse strains. After trio-matings were set-up, mice were plug checked every morning. Plugs signified mating occurred overnight and were treated as potential pregnancies.

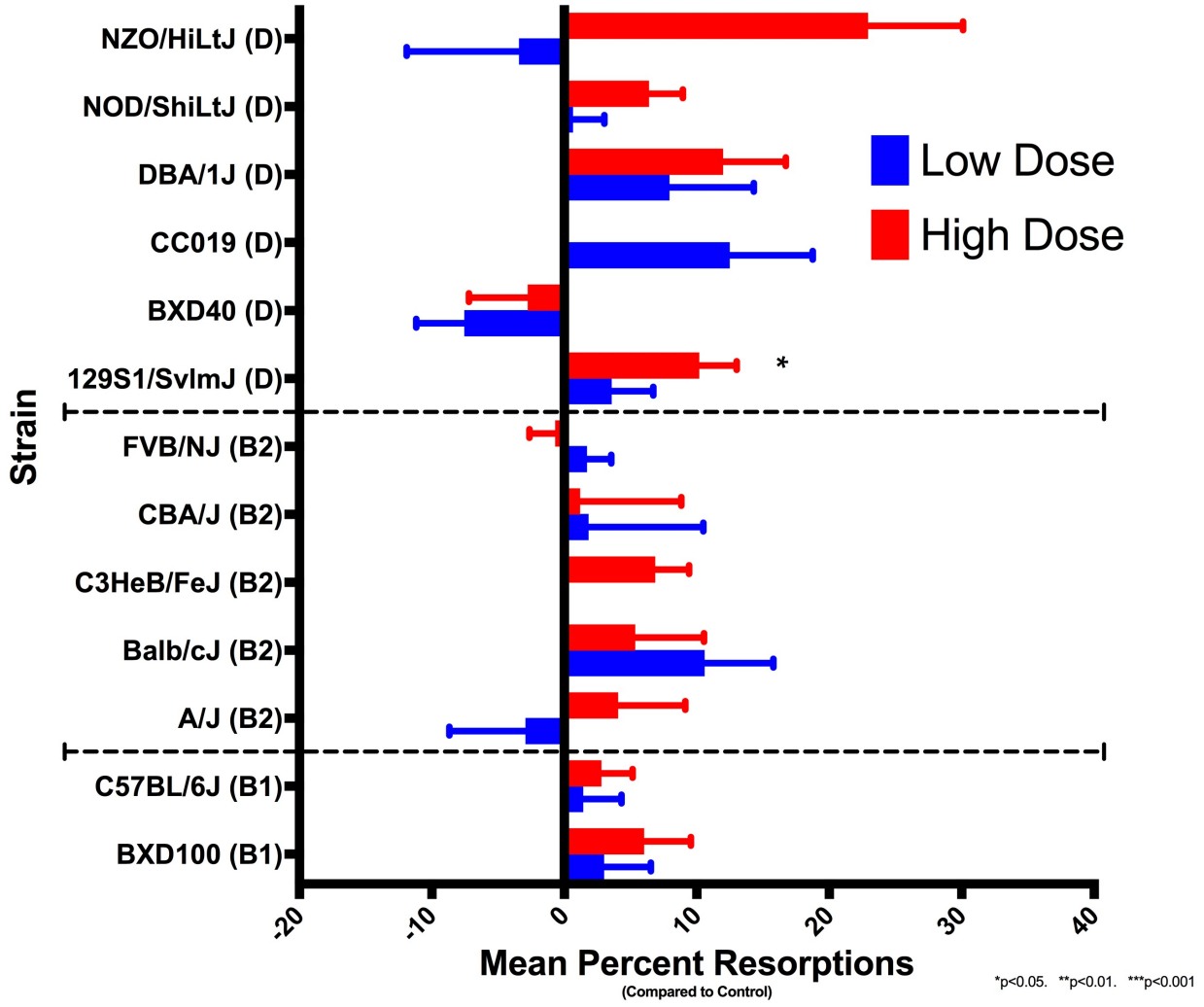


Figure 3-3. Percent Embryos Resorbed Within Implantation Sites Compared To Controls Of The Same Strain. Average percent of non-viable or necrotic tissue found within implantation sites in uterus at gestation day 10.5 (euthanasia) compared to pregnant control groups. Data expressed as mean values ± SEM.

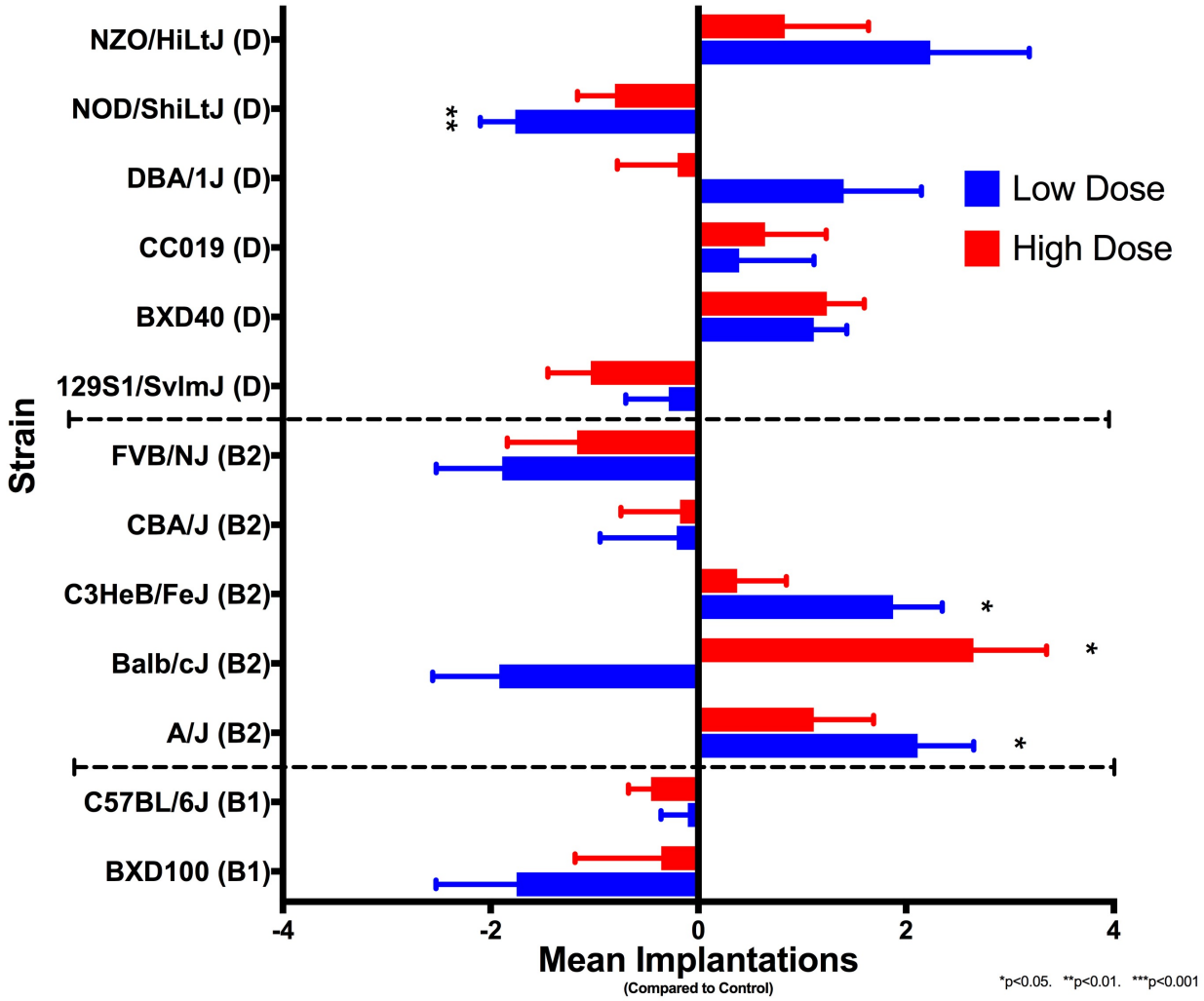


Figure 3-4. Mean Implantation Sites Of Pregnant Mice Compared To Controls Across All Strains. The number of implantation sites found within the uteruses at euthanasia was recorded for each administration group within each strain compared to pregnant control groups. Data expressed as mean values ± SEM.

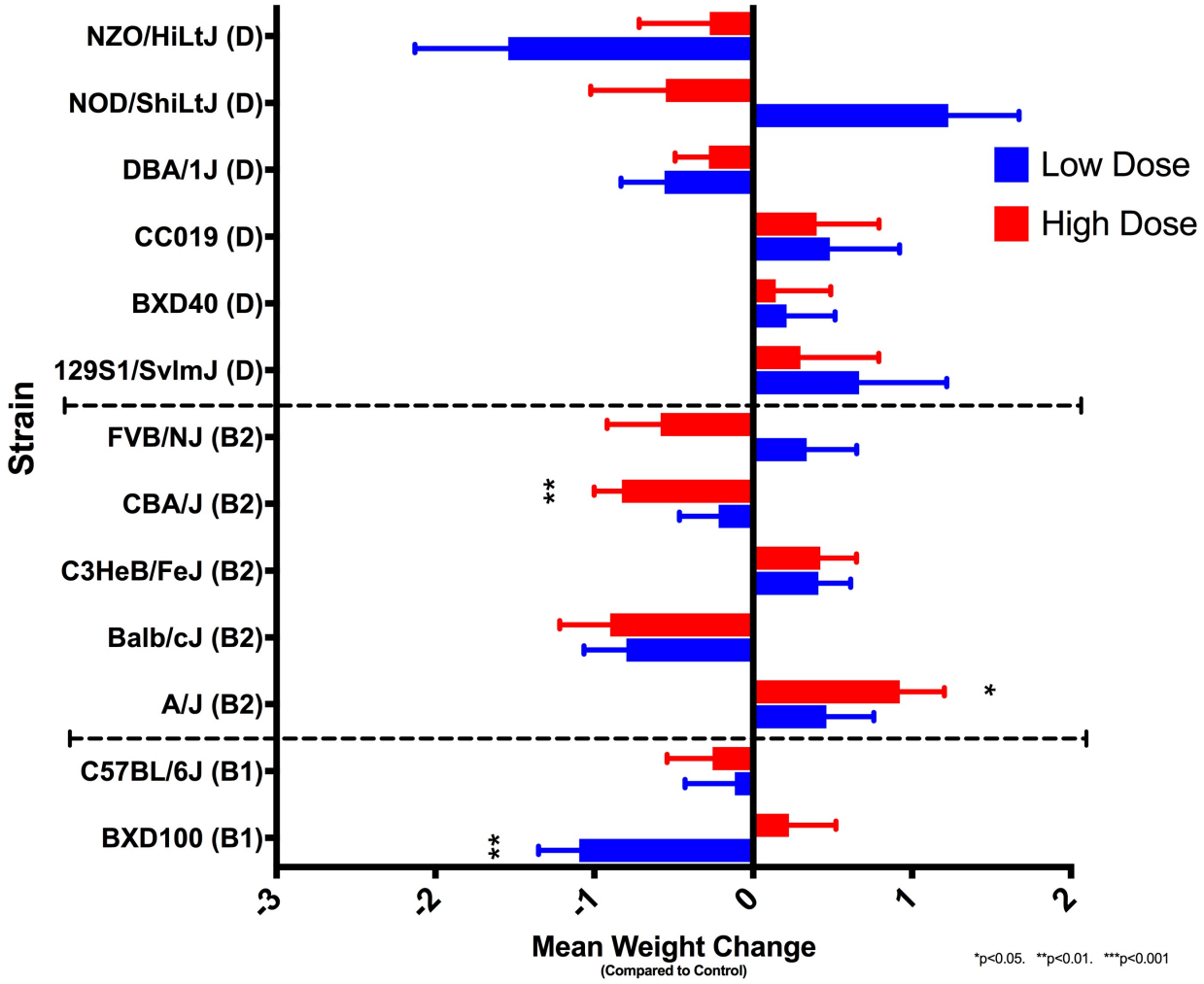


Figure 3-5. Mean Overall Body Weight Change Compared To Controls. Mean change in body weight (g) of pregnant mice between gestation day 1 (copulation plug) and gestation day 10.5 (euthanasia) compared to pregnant controls. Data expressed as mean values ± SEM.

IV RT-PCR QUANTIFICATION OF CARDIOGENIC TRANSCRIPTION FACTORS IN DEVELOPING EMBRYO HEARTS OF DIVERSE MOUSE POPULATIONS EXPOSED TO DIOXIN

IV.1 Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is a potent, ubiquitous environmental toxicant that effects reproduction and development in mammals and fish, which poses risks to human health. Embryos and fetuses of aquatic and mammalian species have shown to be more susceptible to dioxin toxicity. This is of great concern as maternal body burdens are transferred to their offspring during pregnancy and through breastfeeding(Furst, 2006; Schecter, Kassis, & Papke, 1998). Developmental toxicity of dioxin and dioxin-like compounds became evident through children born to mothers who reported decreased birth weights, respiratory distress, and hyperpigmentation of skin.

Cardiogenesis is especially sensitive to toxicity of environmental toxicants, including dioxin, as is the longest functioning organ in the developing fetus. Chick embryo and zebrafish studies have revealed that exposure to dioxin and dioxin-like compounds leads to decreased heart size, valve malformation, and arterial dysfunction, similar to those seen in congenital heart defects of children born to mothers living in areas of high dioxin exposure(Walker & Catron, 2000).

Although molecular and histological aspect of dioxin-induced cardiotoxicity have been largely studied in zebrafish, which shows changes in embryo hearts, intestines and

hepatocytes, developmental studies have not been performed in depth in mammals. Early histological changes in the heart were mostly deleterious, which resulted in impaired function(Antkiewicz, 2005; Mehta, Peterson, & Heideman, 2008). Factors contributing to cardiotoxicity include master regulators and transcription factors associated with normal development and function of the target organ being studied. Understanding how exposure alters the expression of these genes can uncover the underlying fundamental mechanisms of dioxin cardiotoxicity.

Early signaling cascades involved in cardiogenesis are conserved across species. These highly conserved genetic programs are controlled by gene regulatory networks(Davidson & Erwin, 2006). Cardiac master regulators that control the early stages of cardiogenesis, such as GATA4, NKX2-5, AND TBX5, can be detected at the transcription level. Upon exposure to dioxin, early cascades alter the expression levels of downstream networks leading ultimately leading to the overall dysfunction of the heart later in development. Differences in gene expression levels of these factors can vary among individuals and can also play a vital role in determining overall cardiotoxicity.

Dioxin-related toxicities have been described for several mammalian systems, including reproductive, endocrine, nervous, immune and skeletal systems(Yoshizawa, Heatherly, Malarkey, Walker, & Nyska, 2007). The mammalian cardiovascular system has been a known target for halogenated aromatic hydrocarbon (HAH) toxicity(Mohsenzadeh, Zanjani, & Karimi, 2018). Our previous dioxin exposure study examined the effects on genetic variation on maternal endpoints of toxicity. We found that maternal endpoints

were not sensitive enough to identify high individual susceptibility to dioxin toxicity. To investigate markers that are potentially more sensitive, we examined dioxin induced cardiotoxicity in mouse embryos and the expression of several transcription factors involved in cardiogenesis (Table 4-1). The genes chosen are well-known in the early stages in cardiogenesis and should be sensitive markers for response to *in utero* dioxin exposure (Table 4-2).

IV.2 Methods

IV.2.1 Animals

All experimental procedures and protocols for animal use and research were approved by IACUC at Texas A&M University. Inbred mice were obtained from The Jaxson Laboratory (Bar Harbor, Maine) and from the Collaborative Cross mouse resource at the University of North Carolina and bred in house. Mice were provided water and standard mouse chow (Rodent Diet Tekland 8064, Envigo, Madison, WI) *ad libitum*. Mice were set-up in trio matings (2 females and 1 male) and checked daily for copulation plugs to establish embryonic timing; noon on the day that plugs were detected was assigned embryonic day (E) 1. Dioxin doses (beginning on E1) were administered using 3g aliquots of peanut butter (PB) based on initial weight of mice. Peanut butter was made by mixing 140g PB2® powder (Powdered Peanut Butter, Bell Plantation Inc., Tifton, GA) and 200 mL of ultrapure water (Milli-Q Advanced Water Purification System, MilliporeSigma, Darmstadt, Germany). Mice were given their respective dose daily through PB aliquots.

IV.2.2 Cardiac Tissue Collection

At day E10.5 mice were euthanized by CO2 asphyxiation and cervical dislocation for organ and tissue collection. The uterus was removed and further dissected for removal of embryos. After embryo examination, cardiac tissue was further removed from E10.5 embryos and stored in RNAlater (Sigma Aldrich).

IV.2.3 RT-PCR Expression of Cardiogenic Markers

IV.2.3.1 RNA Isolation

Heart tissues were washed in TE Buffer prior to isolating RNA to reduce the interaction of RNAlater with isolation process. Washed tissues were then homogenized in 500mL of TriZOL Reagent (Invotrogen, Carlsbad, CA) with chrome-steel beads using a TissueLyser II (Qiagen). The optional chloroform extraction step was added to the manufacture's protocol. RNA was further isolated using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). The manufacturer's instructions were followed with the optional PureLink DNase Set (Invitrogen, Carlsbad, CA) added. The quality (260/280 ratio) of RNA was analyzed using Cytation.

IV.2.3.2 RT-PCR

Conversion of 2μg RNA to cDNA was done using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). LightCycler 480 SYBR Green I Master Mix was used to

assess the relative expression of *Cyp1A1, Cyp1B1, Gata4, Hand1, Hand2, Mef2c, Nkx2.5, Pitx2, Tbx3, Tbx5*, and *Tbx20*. RTPCR was performed using a LightCycler 96 System (Roche, Basel, Switzerland). Expression was normalized to the geometric mean of *Gapdh* and *β-Actin* for the SYBR green-based analysis. The $2^{-\Delta\Delta CT}$ method was used to calculate fold changes(Livak, 2001).

IV.2.4 Statistical Analysis

Statistical analyses were performed using JMP Pro 12 (Cary, NC) statistical software and graphs were generated with GraphPad Prism 8 (San Diego, CA) software. One-way analysis of variance (ANOVA) tests were conducted on change in relative gene expression of cardiac genes (Table 4-2). If F static showed significance, the Dunnet's t-test for pair-wise comparisons was used for each dosing group. Data were presented at group means $\pm$ SEM. Results were considered statistically significant if $p<0.05$.

IV.3 Results

After 10 consecutive days of dosing mice with 1 ng/kg and 100 ng/kg of dioxin, we quantified the relative expression of transcription factors and genes relevant to cardiac development. Our previous studies, focusing on the reproductive and developmental effects of dioxin exposure showed very little intrastrain statistical significance in response, but significant variations in interstrain responses. We then focused on observing the genetic changes in embryo hearts to identify possible molecular changes in response to

in utero dioxin exposure, specifically expression levels of genes involved in cardiogenesis. Heart data showed overall significant interstrain differences in cardiac gene expression and variations of significant interstrain differences in gene expression. Both data sets emphasize the influence genetic background has on response to acute dioxin exposures. However, the random distribution of significant relative gene expression across strains remains consistent with the random intrastrain significant differences seen in previous reproductive and developmental endpoints.

IV.3.1 Interstrain Variability in Cardiac Gene Expression

We evaluated gene expression for 11 transcription factors that are either involved in cardiogenesis or significant to dioxin exposure (Table 4-2). Relative gene expression values were measured in pooled embryo hearts from individual pregnant mice from our previous maternal studies and used to analyze changes in relative gene expression (Figure 4-1). All genes showed significant interstrain differences at both treatment levels. Intrastrain differences varied across genes and dose level.

IV.3.2 Dioxin-Induced Changes in Cytochrome P450 (CYP) Gene Expression of Embryo Hearts

Common cytochrome P450s (CYPs) expression levels were examined due their known induction in relation to acute dioxin exposures(Carney, Peterson, & Heideman, 2004; Dragin et al., 2006; Nebert et al., 2004). The majority of the significant differences in

Cyp1a1 expression levels are seen in the 1ng/kg dose of dioxin compared to the controls, with mostly no significance seen at the 100 ng/kg dose. This could be a possible result of acclimation, in which fetal hearts become accustomed to exposure at the low dose but show no signs of toxicity at the high dose. *Cyp1b1* expression has a higher instance of interstrain significance seen between the 100 ng/kg dose and the controls. There are fewer significant differences in *Cyp1b1* expression compared to *Cyp1a1*. Some of the mouse strains, including BALB/cJ, BXD100, and CBA/J, showed no changes in CYP expression in response to dioxin exposure. This data shows that changes CYP expression are strain-dependent, and cannot solely determine dioxin toxicity.

IV.3.3 Dioxin-Induced Changes Cardiac Gene Expression of Embryo Hearts

Some mouse strains showed very few significant changes in response to dioxin exposure. Those strains, including CBA/J and NZO/HiLtJ, had very little significant differences in cardiac gene expression, but showed changes in PITX2 and TBX5 expression, which could possibly cause changes in fetal heart looping, chamber formation and septation in the mature hearts of embryos exposed to dioxin *in utero*. Although these changes are expected to be seen in the later stages of cardiogenesis, they could potentially but fatal to the mature heart.

Acclimation is seen across several cardiac genes, mostly in A/J, BXD40, BXD100, and C3HeB/FeJ strains. These strains will mostly like exhibit early and late heart malformations that could become fatal in the mature heart at very low doses of dioxin,

due to the significant changes in expression levels of key regulators of response (such as GATA4 and NKX2.5). Very few significant differences are seen at the 100 ng/kg dose, which yields them less sensitive to toxicity at high doses, with increased sensitivity to low doses. These strains could potentially represent individuals with high susceptibility to the cardiogenic effects of dioxin exposure. Overall, dioxin-induced toxicity is seen across cardiac genes in a strain- and gene-dependent manor. Individual strain susceptibility varies among the measured cardiac genes with no significant patterns or inherent similarities to previous maternal data.

IV.4 Discussion

It has long been known the effects of maternal dioxin exposure. Much of which has been revealed was through various accidental exposures including industrial explosions in Seveso, Italy and the massive herbicidal use of Agent Orange in the Vietnam war(CDC, 1988; Eskenazi et al., 2003). These two incidents alone revealed downstream teratogenic, nephrotoxic and hepatoxic effects of those exposed. Recent studies continue to uncover the teratogenic effects those exposures have on breast milk, fetal death, and the overall development of the children exposed through maternal and environmental means.

Much evidence has been revealed about the effects of dioxin-induced toxicity on cardiogenesis, including its fatal effects on fetal hearts, altered linear heart tube formation and looping, and failed maturation of fetal hearts. Our study not only focused on the

underlying genetic causes of these malformations, but proved the effectiveness of using a genetically diverse mouse population to identify high susceptibility to the toxic effects of *in utero* acute dioxin exposures. The significant intrastrain differences shown across the cardiac genes measured further exerted our claim in our maternal study that individual susceptibility is dependent on both the toxic dose and the endpoint being measured.

The genetically diverse population of mice chosen for this study showed that some strains have higher chances of altered expression in some genes but not in others. For example, 129/SvlmJ mice are more susceptible to expression changes of *Pitx2* and *Tbx20* at both the high and low dose of dioxin, but no effects in *Hand1* expression at either dose. The opposite is seen in NZO/ShiLtJ mice, thus proving the importance of including genetic variation found in the population in toxic exposure studies. Other studies have incorporated the use of genetically diverse mouse populations to improve current risk assessment practices, discover biomarkers of toxicity to acetaminophen and to understand the genetic parameters of benzene toxicity(French et al., 2015; Harrill & McAllister, 2017; Harrill et al., 2009). The concept of these and related studies is that the use of genetically diverse mouse populations is more effective in identifying and understanding models of disease and susceptibility found within certain subpopulations. The use of single, common rodent strains lacks the genetic variation required in identifying key genetic elements of toxicity, thus can be ineffective when compared to rodent population studies.

Several aspects of our project may have improved the methods by which toxicogenomic exposures studies are conducted. We mimicked the diversity found within the general population with the use a diverse inbred mouse strains. Those strains that showed significant changes in gene expression could serve as population-based biomarkers of response. The unique genetic backgrounds of each strain allow us to better define the intrinsic genetic factors involved in susceptibility to dioxin toxicity and similar compounds. With this knowledge, we have the potential to detect high susceptibility within the general population earlier during exposures to toxic chemical substances. This is especially beneficial to pregnant women and developing fetuses, as they are at high risk for developmental defects upon exposure.

IV.5 Conclusions

The teratogenicity of acute, dioxin exposure is of great concern due to its negative outcomes and implications on fetal development. Understanding maternal endpoints alone are not significant enough to gain a full understanding of how genetic backgrounds impacts susceptibility and exposure response. Our study contributes to the understanding that susceptibly depends not only on genetic background, but also the endpoint being studied. We focused on cardiogenic markers in dioxin response, which identified potential strain-dependent genetic markers of toxicity. This data further emphasizes the importance in utilizing genetically diverse mouse populations during toxicity studies.

IV.6 Figures and Tables

PRIMARY STRUCTURE	CARDIAC CRESCENT	LINEAR HEART TUBE	LOOPING HEART TUBE/ELONGATION	CHAMBER FORMATION/SEPTATION	SEPTATION/ MATURATION
HUMAN	Day 15-17	Day 17-19	Day 29	Day 24-36	Day 36 – Birth
MOUSE (EMBRYONIC DAYS)	E7.5	E8	E9	E10-12	E12 - Birth
TRANSCRIPTION FACTORS	GATA4 NKX2-5 PITX2 MEF2C TBX5	GATA4 NKX2-5 PITX2 MEF2C TBX3 TBX5	GATA4 NKX2-5 PITX2 MEF2C TBX3 TBX5 TBX20 HAND1 HAND2	GATA4 NKX2-5 PITX2 MEF2C TBX3 TBX5 TBX20 HAND1 HAND2	GATA4 NKX2-5 PITX2 TBX5

Table 4-1. Comparison Of Human And Mouse Stages Of Cardiogenesis And Genes Expressed. Chart of the primary structures, with corresponding transcription factors expressed at that stage, found at different stages of cardiogenesis in humans compared to mice.

GENE SYMBOL	GENE NAME	MAIN FUNCTION (CARDIOGENESIS)	DESCRIPTION	REFERENCES
Cyp1A1	Cytochrome P450 1A1	Xenobiotic metabolism (i.e. dioxins)	Biotransformation of endogenous and exogenous substances (i.e. toxins, drugs, etc.)	(Abbott, Schmid, et al., 1999; Bozina, 2009; Carney et al., 2004; Nebert et al., 2004)
Cyp1B1	Cytochrome P450 1B1	Xenobiotic metabolism (i.e. dioxins)	Biotransformation of endogenous and exogenous substances (i.e. toxins, drugs, etc.)	(Bozina, 2009; Nebert et al., 2004)
Gata4	GATA Protein 4 (GATA Binding Factor 4)	Early cardiogenic transcriptional regulator	Expressed early in cardiac development and influences cardiac cell fate (i.e. cardiomyocyte differentiation); Key transcriptional regulator of cardiac genes; Septation; Proepicardium formation	(Narita, Bielinska, & Wilson, 1997; Valimaki et al., 2017; Watt AJ, 2004)
Hand1	Hand Factor 1	Ventricle formation	Left ventricle, pericardium and myocardium formation; Heart looping	(Firulli, 2003; George & Firulli, 2019)
Hand2	Hand Factor 2	Ventricle formation	Cardiac crescent, outflow tract, left and ventricle formation; Contributes to heart tube formation	(Firulli, 2003; George & Firulli, 2019)
Mef2c	Myocyte-Specific Enhancer Factor 2	Cardiogenic transcriptional regulator; Linear heart tube formation	Heart looping; Right ventricle formation/maturation; Vascular endothelial cell differentiation; Early marker of cardiac muscle cells	(Edmondson, Lyons, Martin, & Olson, 1994; Lin et al., 1998; Lin, Schwarz, Bucana, & Olson, 1997)
Nkx2.5	NK-type Homeobox Transcription Factor (*tinman* related)	Early cardiogenic transcriptional regulator; Cardiac crescent and linear heart tube formation; Heart looping	Endocardium and myocardium formation; Cardiomyocyte differentiation; Key transcriptional regulator of cardiac genes; Initiation of cardiogenic cascades	(Benson et al., 1999; Lien et al., 1999; Lyons et al., 1995)
Pitx2	Pituitary Homeobox Transcription Factor 2	Left-right heart signaling mediator	Regulates left/right symmetry; Key in determination of heart looping; Regulates signaling cascade in lateral plate mesoderm	(Franco & Campione, 2003; Franco, Sedmera, & Lozano-Velasco, 2017)
Tbx3	T-box Protein 3	Cardiac transcriptional enhancer; Regulate cell fate and proliferation	Septation; Maturation of cardiac conduction system and cardiac outflow tract; Cardiac pacemaker gene program induction	(Cai et al., 2005; Mohan et al., 2018)
Tbx5	T-box Protein 5	Cardiac transcriptional enhancer; Regulate cell fate and proliferation	Septation; Atrium and ventricle formation; Mutations result in Holt-Oram syndrome	(Bruneau et al., 2001; Cai et al., 2005)
Tbx20	T-box Protein 20	Cardiac transcriptional enhancer; Regulate cell fate and proliferation	Early cardiac crescent signaling cascades; myocardium and endocardium development	(Cai et al., 2005)

Table 4-2. Table Of Major Cardiogenic Factors. Chart of major functions and detailed descriptions of major genes/transcription factors involved in cardiogenesis.

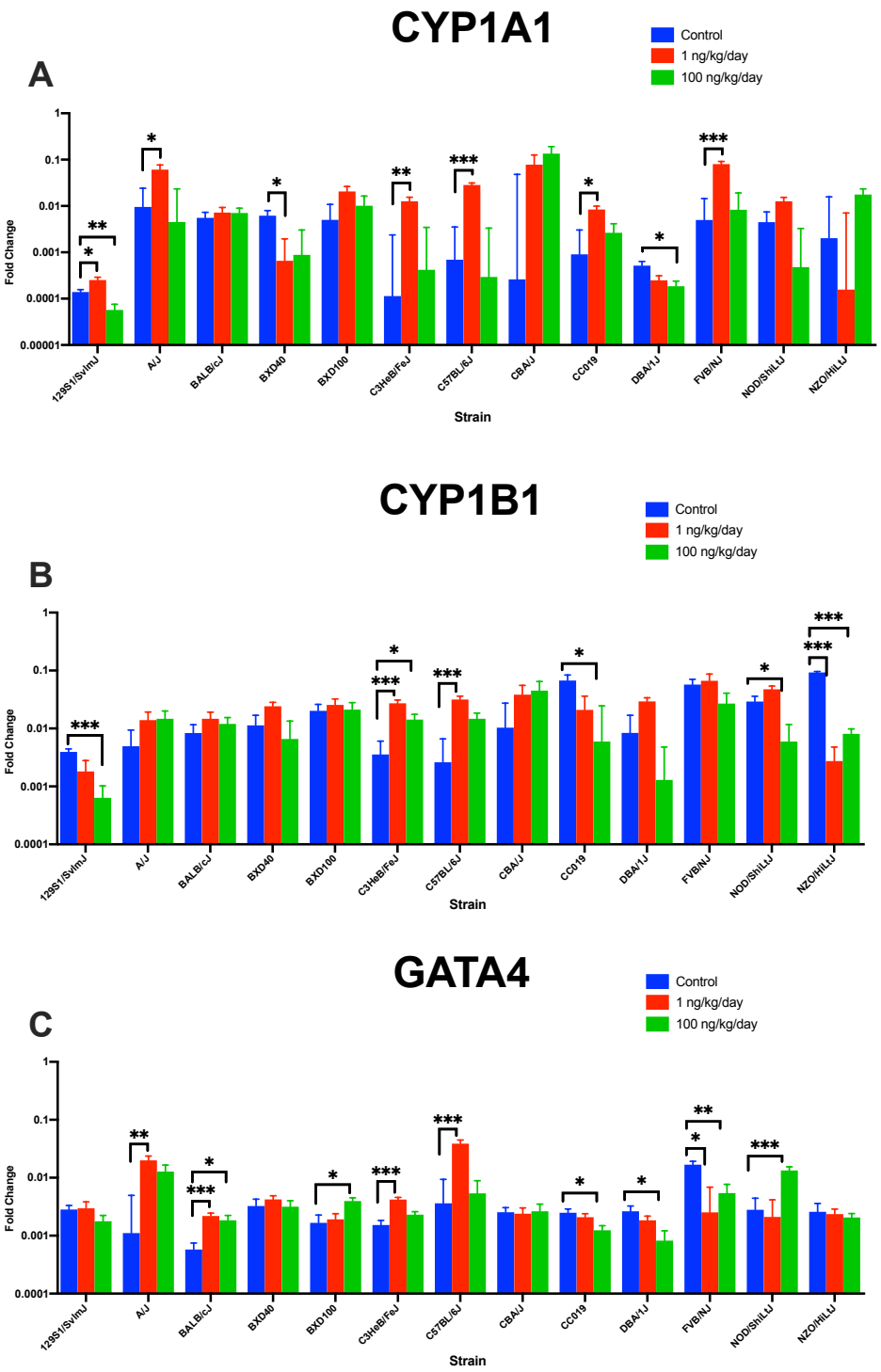


Figure 4-1. Expression Of Cardiogenic Markers In Response To Dioxin Exposure Across A Diverse Mouse Population. Changes in relative expression levels of in (A) CYP1A1, (B) CYP1B1, (C) GATA4, (D) HAND1, (E) HAND2, (F) MEF2C, (G) NKX2.5, (H) PITX2, (I) TBX3, (J) TBX5 and (K) TBX20 in response to low (1 ng/kg/day) and high dose (100 ng/kg/day) dioxin exposures.

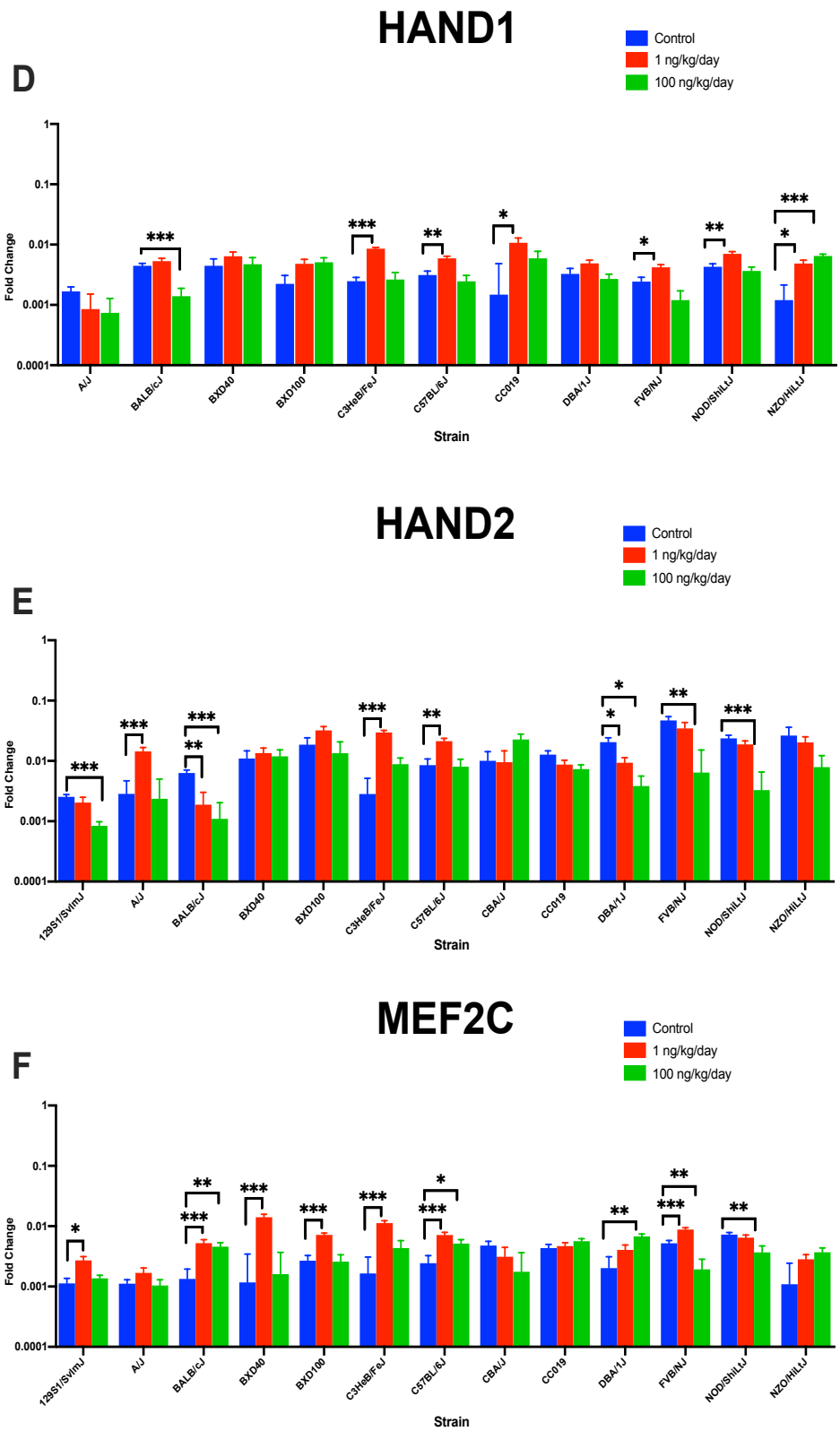
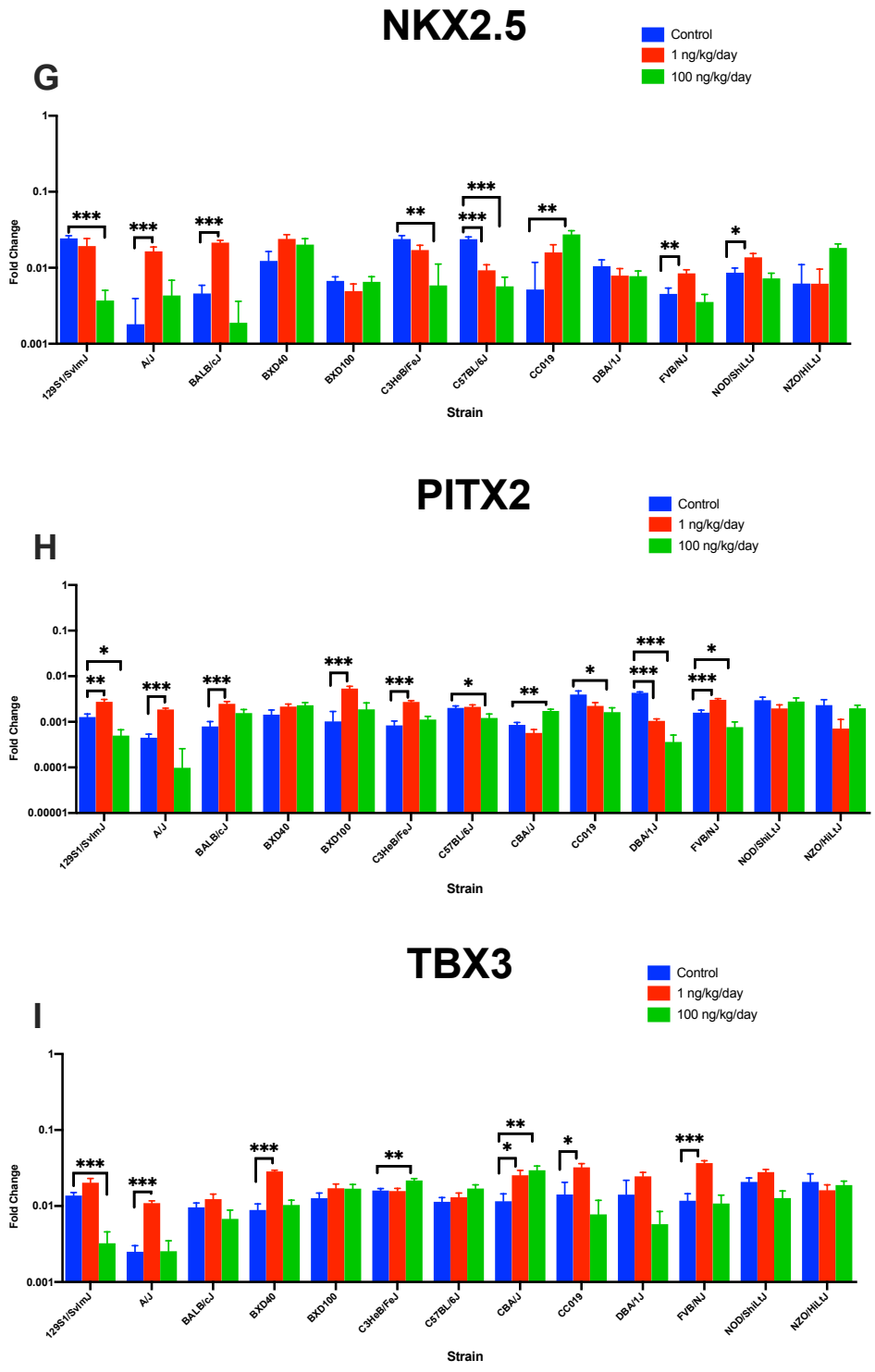


Figure 4-1 Continued



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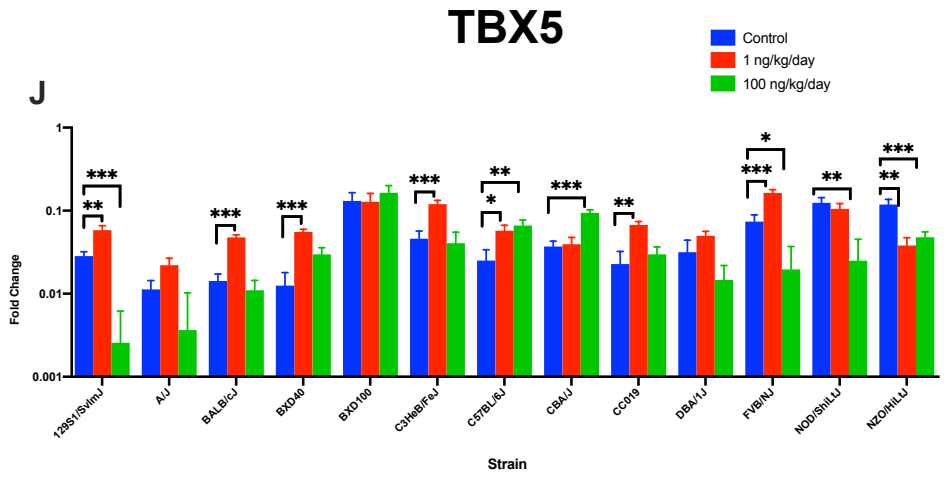

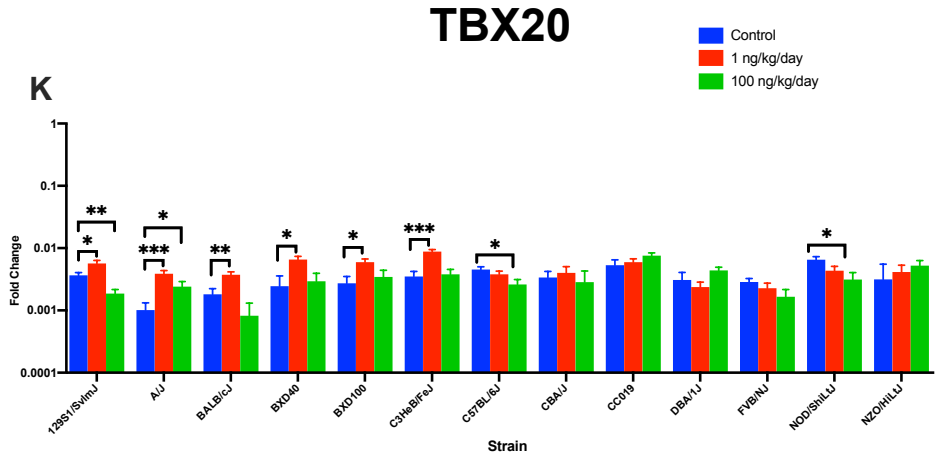

Figure 4-1 Continued

V CONCLUSIONS

V.1 Overview

When assessing environmental risks and depending on the chemical, either cancerous or noncancerous guidelines are followed(Calabrese & Baldwin, 2001; Calabrese, Shamoun, & Hanekamp, 2015). Cancerous guidelines, take on a more aggressive approach to dose-response, that results in linearization of the response curve with no safe level of exposure. With noncancerous guidelines, dose-response curves are believed to have inherent thresholds for a toxic response(Bogdanffy et al., 2001; Bogen, 2016; Calabrese et al., 2015) Exposure levels below the threshold represent “safe” exposure levels in which the general population is expected not to experience adverse outcomes. These levels do not account for uncertainty factors that attribute to individual susceptibility and response variability. By omitting, highly susceptible individuals in the general human population are no considered during the decision-making stages of risk assessment.

Dioxin is ideal for characterizing the utility of a unified approach to noncancerous and cancerous risk assessment guidelines. Unification of these guidelines can decrease the uncertainty found within the available human and animal exposure data, which generally does not include genetic background consideration in response to dioxin. The effects of dioxin have been long studied in both human and animal models, with many noncancerous effects having been uncovered. Animal models has shown that dioxin exposure can result in cancer, but also nephrotoxicity, reproductive and developmental

toxicity, and hepatoxicity(Cole et al., 2003). With the use of its reproductive and developmental toxicity data, in combination with a model that considers genetic heterogeneity, we further expand upon current knowledge with how known effects vary in a heterogeneous population. Our studies help acknowledge sensitive genetic backgrounds found within “safe” exposure levels.

Dioxin and dioxin-like compounds are stable, hydrophobic compounds found throughout the environment(Hay, 1981; Schecter et al., 2006). Humans are commonly exposed to these compounds primarily through contaminated poultry and dairy products(Charnley & Doull, 2005; Schecter et al., 1994), although acute, high dose exposures can occur through industrial accidents. Once exposed, dioxins are stored in adipose tissues due to their hydrophobicity(Byard, 1987). Toxicity is mediated through interactions with the aryl hydrocarbon receptor (AHR), which elicits downstream toxic responses through genomic and non-genomic AhR interactions(Beischlag et al., 2008; Bock Karl & Köhle, 2009; Poland & Glover, 1974). AHR is expressed throughout the reproductive tract of females, which makes dioxin toxicity of heightened concern for reproduction and development. Confirming the sensitivity of these tissues, exposure to dioxin is known to result in various developmental malformations in animal studies, including cleft palate, fetal death, congenital heart defects, and decreased birth weight(Abbott & Birnbaum, 1991; Abbott, Held, et al., 1999; Aragon et al., 2008; Birnbaum, Weber, Harris, Lamb, & McKinney, 1985; Carney et al., 2006; Dragin et al., 2006; Nebert et al., 2004). Our research utilizes the current knowledge of the reproductive effects of dioxin to investigate and understand

how genetic variability may alter susceptibly to reproductive and developmental dioxin toxicity.

Human risk assessors have gaps in the available data that limits their ability to assess exposure risks across populations. Many factors can introduce inherent variability that increases uncertainty. Individual factors, such as age, gender, race, disease state, genetic predisposition, and body composition, are variables that can increase uncertainty when assessing population response variability to toxic compounds(Ashauer, 2010; Chiu, Okino, & Evans, 2009; Dorne & Renwick, 2005). The research reported in this dissertation helps to understand how uncertainty in dose response is generated by genetic variation, and the importance of including factors, like genetic variability, into risk assessment guidelines.

Few research studies have used heterogeneous animal populations to model genetic variation found within the general human population(Dorne, 2010; Dorne, Walton, & Renwick, 2005; Smith, 1996; Walton et al., 2001). Animal models are more suitable for understanding toxicity and dose response to various compounds since they allow us to control for confounding variables, such as disease state, previous environmental exposures and genetic predisposition, that contribute to uncertainty in human data. Mouse models offers several advantages over other animal models. Mice have shorter generation times, produce large numbers of offspring, and are cost efficient, which allow them to facilitate large scale studies. The majority of the mouse genome and general physiology is shared with humans, which provides valuable information on gene function

that is important in understanding mechanistic causes of human disease, biological elements of toxicity, and the development of targeted genetic treatments. Our study utilizes the advantages of mouse models in understanding dioxin response at the population level.

Although there are many advantages in using mouse models, there are also several disadvantages that can confound results. One of the major disadvantages is that animals can be easily stressed during experimental procedures. In fact, mice are typically exposed to chemical compounds through orogastric gavage. This method utilizes a gavage needle the allows a specified dose of a chosen compound to be directly administered into the stomach modeling oral exposure. To reduce the impact of stress of toxicity endpoints, we used the treat method as an alternative dose delivery and highlight the strain-dependent disadvantages of using orogastric gavage as mean of dose delivery during pregnancy exposure studies.

Mice are especially unique for studies involving dioxins, as they have different *Ahr* alleles. They can have either the Ahr^b allele, which is considered “more responsive” or the Ahr^d allele, which is considered “less responsive” and more similar to human *AHR(Harper et al., 1991)*. With these additional allelic differences, mice are more suitable models for human dose response and understanding how genetic variability affects susceptibility. With reported differences in human AHR, mice with the Ahr^b allele become useful in representing sensitive individuals within the population. This increases the inherent

variability within the mouse population to better model human exposure risks associated with dioxin.

Clinical research primarily focuses on the noncancerous effects as they relate to human outcomes during risk assessment(Bogen, 2016; Calabrese & Baldwin, 2001; Gaylor et al., 1999). Since noncancerous protocols follow the threshold approach of dose response, much is unknown about the effects of dioxin on highly susceptible individuals(Dorne et al., 2005; Walton et al., 2001). We show that even for noncancerous endpoints, susceptibility to toxic compounds has a great deal to do with genetic background.

V.2 Findings

In this dissertation, we examined how experimental design alters experimental results and how genetics influences adverse outcomes during hazardous chemical exposures. We examined a standard delivery method (orogastric gavage) and found unique interstrain differences in the stress imposed by orogastric gavage compared to the treat (peanut butter) method during pregnancy. While orogastric gavage can be an effective method in some mouse strains, other strains demonstrated varying responses to stress-related conditions. As pregnancy is already known to be a sensitive endpoint, additional stressors can confound sensitive endpoints like pregnancy. Pregnant 129S1/SvImJ mice had significantly higher serum corticosterone levels, a key marker of stress, following 10 consecutive days of gavage administration compared to peanut butter treats. This confirms that orogastric gavage is an external stressor which was shown to alter

experimental endpoints. In contrast, C57BL/6J mice were more resistant to external stress at all end-points and did not demonstrate an increased stress response in gavage vs treat delivery. The dioxin delivered through the peanut butter method induced an overall higher level of dioxin-induced adverse responses at most endpoints in 129S2/SvlmJ mice than with orogastric gavage. The unwanted stress of gavage can hinder the full effect of exposure and results become difficult to interpret and less reliable for human risk assessments of similar environmental compounds.

Previous research showed that there is an increase in the underlying stress of the animals when the orogastric method is used compared to diet or treat methods of dose administration(Arantes-Rodrigues et al., 2012; Balcombe et al., 2004; Brown et al., 2000; de Meijer et al., 2010). Alternative exposure methods have been suggested, such as the treat method, in which the chemical compound is combined with the treat. Although much has been uncovered on how the stress of experimental conditions impacts experimental results, there is no data addressing how genetic backgrounds influence responses to stressors. Our research confirmed that orogastric gavage causes gavage-induced stress on experimental animals, but in a strain-dependent manner, suggesting that genetics influences the impact of the delivery method. Dose delivery-associated stress and genetic background interact in a way that alter the response data in some strains of mice.

Knowing how individual susceptibility impacts dose-response curves, it should be included when calculating safe exposure levels during risk assessment of individuals exposed to toxicants such as dioxin. Although much is known about potential adverse

effects of dioxin in the environment, much still needs to be discovered concerning its effects across individuals. Determining these effects will ultimately contribute to a better understanding of exposure risks during pregnancy and development.

With the use of the treat method, we exposed a genetically diverse mouse population to dioxin in order to understand the interactions of genetic background and exposure response. We then found that effects of dioxin exposure during pregnancy is largely dependent on genetic background. We analyzed several pregnancy-related endpoints, such as pregnancy rate, implantation, resorption, and maternal weights. Each endpoint showed interstrain variation in response to dioxin. Some strains seemed to be more susceptible to low dose (1 ng/kg/day) than high dose (100 ng/kg/day), while other strains showed no susceptibility to dioxin exposure at all endpoints. The overall data suggest that genetic background contributes to individual susceptibility of dioxin-induced adverse health outcomes.

Additionally, we expanded this study beyond reproduction phenotypes by examining embryonic development, specifically cardiogenesis, at the molecular level. Literature suggests that adverse cardiac effects will be cause by dioxin exposure, but do not assess how genetic background influences heart toxicity. The heart is one of the first major organs to form and function on its own, therefore it is exposed to toxic compounds for a longer time compared to other developing organs. Our data showed that using a heterogeneous mouse population, we were able to identify potential cardiac markers of

susceptibility to early exposure of dioxin. With this data, we hope to achieve a more sensitive readout of dioxin toxicity by identifying potential cardiogenic phenotypes.

V.3 Significance

The use of a genetically diverse mouse population is useful in many studies used throughout risk assessment procedures and guidelines(Harrill & McAllister, 2017). Understanding how underlying doses of hazardous chemical compounds affect essential biological processes is key in exposure assessment. There is limited data on human assessment, which makes using population-based mouse models ideal for estimating internal toxic doses. Mouse data used in this way allows risk assessors to quantitatively address interindividual variability of toxic chemical exposures(Ashauer, 2010; Dorne et al., 2005; Harrill & McAllister, 2017).

Our research investigated the effects of genetic background on response to dioxin exposure during pregnancy. This study broadens the scope of genetic background by utilizing 14 diverse mouse strains. These strains also included allelic differences in the *Ahr* gene, which are known to contribute to variation in dioxin-induced toxicity, allowing us to explore the effect of *Ahr* within a larger genomic context. This is significant in ensuring proper protocols and guidelines are being followed and protecting sensitive individuals within the exposed general population and hazardous occupational settings. Previous research suggests that diverse mouse populations, such as this one, can potentially redefine dose-response with more accurate concentration thresholds (i.e.

linearization of response curve) that can protect more people from adverse health effects of toxic chemical compounds.

Population response variability found within mouse populations are similar to the variation seen within human populations of previous toxic exposure studies(Chiu et al., 2009). Our research showed that the interstrain differences determined the extent to which dioxin induced negative responses at certain endpoints. Single-strain studies have the potential to miss adverse outcomes seen in human subpopulations with higher exposure sensitivity. Our mouse population model provides a means by which genetically sensitive individuals can be detected and incorporated into risk analysis. Our results emphasize the benefits of being able to quantifying the distribution and extent of adverse health effects and define sensitive human subpopulations. In doing so, the mouse population model has the potential to redefine the dose response curve of noncancerous endpoints and guidelines.

Our research is significant to the development of preventative mechanisms and treatment options of conditions that arise during pregnancy in response to toxicant exposure in humans. Upon development of these models, we expect advancements in assessing exposure risks and lessening the environmental burden of dioxin-related complications that affect millions of exposed pregnant mothers residing in highly contaminated areas in the US and around the globe. It also provides new insights on the role of genetics in response variance of individuals exposed to dioxin and similar contaminants.

V.4 Future Directions

The main limitation of this study was the limited number of pregnant females due to low fecundity rates, which limited our statistical power. Mice mate at vastly different rates and do not guarantee mating will occur. These limitations can decrease the potential genetic variability of the mouse population, as strains with low reproductive performance are not usable. In efforts of resolving this problem, we chose mouse strains with medium to high fecundity rates.

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