EARLY LIFE CHEMICAL EXPOSURES AND LATENT MAMMARY GLAND EFFECTS IN MALE AND FEMALE RATS

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ABSTRACT

Adam J. Filgo: Early life chemical exposures and latent mammary effects in
male and female rats
(Under the direction of Suzanne E. Fenton)

Volatile organic compounds (VOCs) are common industrial solvents used in a
number of cleaning agents and solvents. In the United States, spikes in birth defects, infant
mortality, reproductive cancers and leukemia have occurred in areas with high levels of
VOCs in drinking water. My thesis is focused on the effects of a VOC mixture on mammary
gland development and risk for mammary tumor formation following prenatal/perinatal
exposures in a 7,12-Dimethylbenz(a)anthracene (DMBA)-induced mammary tumor rat
model. To gain an understanding of the animal model used in my studies, the Harlan Sprague
Dawley (HSD) rat, an atlas of mammary gland development was created for both sexes,
starting at embryonic day 15.5 through postnatal day (PND) 70. In addition, prenatal
exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin and diethylstilbestrol were used to
demonstrate delayed and accelerated mammary gland growth, respectively. This atlas of
mammary gland development in the HSD rat will be a key component in understanding and
interpreting effects of chemicals in this rat strain. To specifically test the VOC mixture in this
rat model, time-pregnant HSD rats and their offspring were given access to water containing
a mixture of VOCs at concentrations 5X, 10X and 50X times those detected in contaminated
drinking water at Camp Lejeune, to determine VOC body burden in both dams and pups.
Mammary gland development was found to be accelerated in both sexes at the 5X and 10X concentrations. DMBA was given to VOC-exposed animals at PND 30 and tumors were recorded and collected 26 weeks after carcinogen treatment. There was a significant trend increase in adenocarcinomas arising in a fibroadenoma with VOC exposure in the females. Additionally, two malignant tumors formed in the males in the 10X exposure group and the males that died early due to tumor burden were from the 10X exposure group. These data demonstrate the ability of the VOC mixture to accelerate mammary gland development and enhanced the risk for tumor formation following carcinogen exposure. The mixture of VOCs was active at only 5-10 fold higher levels than what Marines or their mothers might have consumed, and these low level, prenatal exposure effects deserve further attention.
ACKNOWLEDGEMENTS

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In addition, I would like to thank all my fellow labmates at the NIEHS who have become a part of my second family in North Carolina. Their aid and friendship has helped me throughout my graduate studies. I would especially like to express my deepest gratitude and thanks to my advisor and role model, Dr. Suzanne Fenton. Her enduring encouragement and practical advice has provided an excellent atmosphere for doing research.

Finally, I would like to acknowledge the abundant support and patience of my friends and family. I would like to explicitly thank Linda and Greg Filgo, Julie Bruno, Dan McLeod and Shawn Dayson Shifflett for their love and unwavering faith in me through the years. They, and so many others, have been a vital source of happiness, both in and outside of graduate school.
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<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ATSDR</td>
<td>Agency for Toxic Substances and Disease Registry</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CRSD</td>
<td>Charles River Sprague Dawley</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P-450</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetic acid</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine disrupting chemical</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERα/β</td>
<td>Estrogen receptor alpha/beta</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GD</td>
<td>Gestational day</td>
</tr>
<tr>
<td>GHR</td>
<td>Growth hormone receptor</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat-induced epitope retrieval</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HSD</td>
<td>Harlan Sprague Dawley</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>IGFR</td>
<td>Insulin-like growth factor 1 receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>LAU</td>
<td>Lobuloalveolar unit</td>
</tr>
<tr>
<td>LE</td>
<td>Long Evans</td>
</tr>
<tr>
<td>MCL</td>
<td>Maximum contamination level</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>NIEHS</td>
<td>National Institute of Environmental Health Sciences</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NMU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>NTP</td>
<td>National Toxicology Program</td>
</tr>
<tr>
<td>PERC</td>
<td>Tetrachloroethylene</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid-phase microextraction</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TDLU</td>
<td>Terminal ductal lobular unit</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCE</td>
<td>Trichloroethylene</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>trans-1,2-DCE</td>
<td>trans-1,2-Dichloroethylene</td>
</tr>
<tr>
<td>VC</td>
<td>Vinyl chloride</td>
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<tr>
<td>VOC</td>
<td>Volatile organic compound</td>
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CHAPTER 1: Introduction

My thesis work has focused on a human health problem that became known in the popular press. It was based on a 30-year contamination event that left many middle aged male Marines and the children of Marines wondering why they may have been diagnosed with breast cancer.

Breast cancer is the most commonly diagnosed cancer in women, and after lung cancer, is the second most common fatal cancer of women [1]. In 2014, there was an estimated 232,670 new cases of invasive breast cancer diagnosed in women in the United States, which equates to about 1 in every 8 women [2]. The peak age range for diagnosis of breast cancer in women is 55-64[1]. In 2004, there was a decrease in the breast cancer incidence rate of almost 7% due to reductions in use of hormonal replacement therapy in post-menopausal women. Between 2006 and 2010, breast cancer incidence rates have remained stable and deaths from breast cancer have steadily decreased [3]. Breast cancer in men is uncommon and accounts for about 0.5-1% of total breast cancers [2]. In 2014, less than 1 in 1000 men developed breast cancer with an estimated 2360 new cases in the United States [3, 4]. Unlike breast cancer in women, male breast cancer incidence peaks at 71 years of age and the incidence rates may be slowly rising [5, 6]. It has been estimated that fifteen percent of breast cancers in men are familial [6]. Most diagnoses of breast cancer, in both men and women, are without associated breast cancer susceptibility gene mutations.
Known risk factors for women include: family history, genetic variants (BRCA1, BRCA2, TP53, PTEN, etc.), breast density, history of benign breast disease, reproductive factors (increased lifetime exposures to estrogen and progesterone and not having children), physical activity, alcohol consumption, radiation exposure, and adult body mass [7, 8].

Potential risk factors for men include; family history, estrogen excess (medication, Klinefelter syndrome), genetic variants (similar to those in women), occupational exposures to radiation, carcinogens and potentially volatile organic compounds [4, 6]. Interestingly, environmental and lifestyle factors contribute to 70–95 % of breast cancer risk [9]. Even familial breast cancer risk is speculated to be the interaction between lifestyle factors and common gene variations (that only add a small increase in breast cancer risk) [3].

Exogenous chemical exposures (aryl aromatic amines, N-nitrosamines, tobacco smoke, etc.) have had significant correlations with risk for breast cancer development in epidemiological studies around the globe [8]. However, it is difficult to prove causation because the disease is often diagnosed decades after environmental exposures and humans are not exposed to one chemical. Even when causation may be attributed to a particular environmental exposure, such as in rodent model studies in a controlled environment, it is often complicated to determine a mechanism by which it affects the mammary tissue due to the complexity of the gland and its intricate signaling pathways. There are windows of susceptibility in mammary gland development, not common to other organs, when exogenous chemical exposures could influence breast development and increase breast cancer risk [7].

The mammary glands of both humans and rodents undergo four phases of critical growth and development; in utero, neonatal, puberty and pregnancy followed by involution.
Little is known about the mechanism of fetal breast formation in humans, but in rodents mammary gland development involves communication between the epidermis and mesenchyme by various signaling pathways [10, 11]. The known signaling events of rodent embryonic female mammary gland development from milk line formation to rudimentary ductal tree present at birth have been described [12]. The milk line requires early Wnt signaling and leads to the formation of the mammary placode, a thickening in the epithelial layer and the first sign of mammary gland formation [13]. The rodent mammary bud then forms due to a number of signaling peptides expressed within either the epithelial or mesenchymal cells including; Wnt 11, β-catenin and sex steroid hormone receptors like estrogen receptor α/β (ERα/β) and androgen receptor (AR). The rudimentary ductal tree development from the mammary bud is known to be hormone independent, while regulation of the initial phase of ductal growth is not understood [12]. Male and female rodents have morphologically different mammary glands in utero, while human mammary glands are not sexually dimorphic until puberty. Male mice have a two day surge of fetal androgens at embryonic day (E) 13.5-15.5 that causes apoptosis of the mammary epithelium and regression may eliminate the mammary epithelium [14]. In male rats, due to the androgen surge that occurs around E17 the epithelial bud becomes separated from the epidermis and no nipples are formed, but the rudimentary ductal tree is intact [15]. In humans there is evidence that maternal hormones play a part in growth and development of the mammary glands of fetuses and newborns [16]. While not found in rodents, lobular structures have been seen in newborn infants, and are thought to be a transient structure that dissipates weeks after birth [17].
Before the onset of puberty, isometric mammary gland growth continues in both rodents and humans, independent of hormones [18]. Allometric, or exponential, mammary growth during puberty is dependent on several hormones and paracrine signaling. In rodents, allometric growth is initiated by ovarian steroids. Estrogen and the growth factors triggered by ER activation (epidermal growth factor, growth hormone and insulin-like growth factor-1) signal ductal elongation. Ductal branching and alveolar budding are signaled by progesterone, prolactin, and thyroid hormone [14, 19]. Some genes that are known to control mammary growth during puberty include: inhibin B, colony stimulating factor-1, the progesterone receptor (PR), and Wnt [19]. In humans, regulation of allometric growth is less clear. Acini form on the terminal ducts and embed in intralobular stoma to form terminal duct lobular units (TDLUs), which are the functional units of the breast [17]. Both endocrine and paracrine signaling may also stimulate mammary gland growth in humans, but specific effects are uncertain [14]. Male mammary glands in humans normally do not develop further during puberty [17]. During pregnancy, alveolar proliferation and full differentiation takes place in the rodent mammary gland due to progesterone, placental lactogens, prolactin, osteoclast differentiation factor and the Jak2-Stat5 signaling pathway [19]. In both humans and rodents there is an increase in the number of lobules and a loss of fat in adipose cells of the mammary gland during pregnancy and lactation [17]. Lactational involution of the mammary gland occurs after weaning of the infant. After weaning, the TDLUs decrease in number and size, but the ducts are not involved. During the type of involution that occurs with aging (and not lactational), both the TLDUs and the ducts are reduced in number and are replaced with collagen and fat [17]. In rodents, tissue remodeling and cell death during lactational involution are controlled by Stat3, bax, and bcl-x [19]. The mammary gland is one
of the few truly dynamic organs and undergoes many periods of proliferation and remodeling. For this reason, carcinogens and endocrine disruption chemicals (EDCs) are thought to target the mammary gland. The timing and length of these growth periods may vary across species and may affect carcinogen susceptibility. Some of the potential variations between strains of the rat species will be addressed in Chapter 2, as it is important to understand the animal model for translation to humans. Further investigation of the susceptibility of the mammary gland during periods of growth and development is needed to understand lifelong breast cancer risk.

The negligence in examination of the potential role of the environment in risk for breast cancer in men took a turn in 2009. CNN reported that 20 people, all Marines or sons of Marines based at Camp Lejeune, had been diagnosed with early life male breast cancer and that they blamed chemicals in contaminated well water [20]. The Marine Corps Base, Camp Lejeune, located in Jacksonville, North Carolina is the site of one of the worst ground water contamination events in U.S. history. Between 1953 and 1987, ground water wells were contaminated with volatile organic compounds (VOCs) from off-base ABC One Hour Cleaners (a dry cleaning facility), on-base metal degreasing and/or leakage from underground fuel storage. Among the 70 contaminates measured in the well water, 1500 parts per billion (ppb) of tetrachloroethylene (PERC), 50 ppb of trichloroethylene (TCE), 100 ppb of trans-1,2-dichloroethylene (trans-1,2-DCE), 25 ppb of vinyl chloride (VC) and 50 ppb of benzene were detected in the water supply[21]. These five VOCs are examined in this dissertation for their potential effects on mammary gland development and susceptibility to cancer. The Agency for Toxic Substances and Disease Registry (ATSDR) reports that compared with the Camp Pendleton workers in California, who were not exposed to VOCs, the Camp Lejeune
workers had higher mortality rates for leukemia, multiple myeloma, Parkinson’s disease, kidney disease and cancers of the female breast, kidney, lung, oral cavity, prostate, and rectum [22]. Additionally, at least 84 men living or serving at Camp Lejeune developed invasive breast cancer [23]. Environmental exposure to VOCs during windows of susceptibility could be an important environmental factor in breast cancer risk. As low dose, mixture studies of VOC exposure during mammary gland development have not yet been preformed, the overarching goal of this dissertation project was to evaluate the role of an environmentally relevant mixture of VOCs, given during critical windows of susceptibility, on mammary gland development and later susceptibility to cancer in male and female rats. In Chapter 2, the mammary gland development of the Harlan Sprague Dawley (HSD) rat is extensively evaluated, in both the male and female. Defining this rat model provides an aide in toxicological assessment of chemicals on the mammary gland. Chapter 3 sets out to better understand VOC exposure in sensitive subpopulations of rats; namely the pregnant mother and her development offspring. Chapter 4 addresses the key question of whether early life VOC exposure alters mammary gland development or affects vulnerability to carcinogens. In Chapter 5, the novel findings, limitations, impacts on science and future studies are summarized and further discussed.

**Background on Volatile Organic Compounds (VOCs)**

VOCs have become common ground water contaminate due to their physical and chemical properties that allow them to easily move between air, soil and water [24]. VOCs are a family of organic chemicals that usually have high vapor pressure, low to medium water solubility and low molecular weight. VOCs are formed naturally, industrially, or sometimes both [24, 25]. Of the five VOCs of interest in this dissertation all but DCE have
been extensively studied by the National Toxicology Program (NTP), U.S. Environmental Protection Agency (EPA) and the International Agency on Cancer Research (IARC). The following background information is a compilation of data from their risk assessment papers; NTP Report on Carcinogens, EPA Support of Summary Information on the Integrated Risk Information System and IARC Monographs.

**Benzene**

Benzene is an aromatic compound and ranks in the top 20 in production volume for chemicals produced in the United States. At room temperature, it is a colorless liquid that is only slightly soluble in water. It has a sweet odor and is highly flammable. Benzene can occur naturally; sources include gas emissions from volcanos and combustion events such as forest fires and the burning of tobacco. Benzene is also produced in large quantities and is used as a solvent, to synthesize other industrial chemicals, and in a variety of products like rubber, drugs and pesticides [24, 26, 27]. Structural, chemical and physical properties are shown below.

![Chemical structure of benzene. Obtained from a Google image search.](image-url)

Figure 1.1. Chemical structure of benzene. Obtained from a Google image search.
Table 1. Chemical and physical properties of benzene[27]

<table>
<thead>
<tr>
<th>Property</th>
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<tbody>
<tr>
<td>Molecular weight</td>
<td>78.1 g/mol</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>0.8787 at 15°C/4°C</td>
</tr>
<tr>
<td>Melting point</td>
<td>5.5°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>80.1°C</td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>2.13</td>
</tr>
<tr>
<td>Water solubility</td>
<td>1.79 g/L at 25°C</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>94.8 mm Hg at 25°C</td>
</tr>
<tr>
<td>Vapor density relative to air</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Exposure**

Benzene is ubiquitous in the atmosphere and has been identified in the air, water or soil over 1,000 hazardous waste sites [26]. Benzene is released into the environment naturally, but more importantly through anthropogenic emissions such as gasoline vapors, auto exhaust and chemical production. Benzene is chemically degraded in a matter of hours to days in the air, primarily due to reactions with naturally occurring hydroxyl radicals. Volatilization, photo-oxidation, and/or bacterial-biodegradation of benzene in the soil and water can break it down in a few weeks under aerobic conditions or several months under anaerobic conditions [26, 28]. Benzene has been detected in ground water samples in 832 of the 1684 National Priorities List sites [26]. Sources of benzene in ground water include underground fuel storage leaks, accidental spills and leaching from contaminated soils[26, 28].

The primary route of human exposure to benzene is inhalation of tobacco smoke and ambient air, with higher exposures in areas of high vehicle traffic and gas stations. Benzene is readily absorbed via inhalation, oral and dermal exposure. Benzene is detected in the blood of 100% of smokers and 45% of non-smokers with a mean concentration of 0.136 µg/L and
0.024 µg/L, respectively [29]. From the blood, benzene quickly distributes throughout the body and tends to accumulate in fatty tissues. The liver is the major site of metabolism and production of several reactive metabolites. Benzene can also cross the placenta and enter the cord blood where it has been detected at similar levels as found in the mother’s blood [26, 28].

**Toxicity**

There are many known human health effects caused by benzene inhalation. It is well established that benzene can cause hematological, dermal, immunological, neurological and reproductive effects in humans. However, the most significant effect in humans is cancer; acute non-lymphocytic leukemia, non-Hodgkin’s lymphoma and multiple myeloma, in particular [26]. There is no information on the oral carcinogenicity of benzene in humans, but benzene has been shown to be a multiple site carcinogen in chronically adult-dosed animals. In adult rats receiving 25-200 mg/kg/day of benzene by gavage in corn oil, there were significant increases in Zymbal gland carcinomas, oral cavity squamous cell papilloma and carcinomas and skin squamous cell papilloma and carcinomas. In chronically exposed adult mice, orally-administered benzene caused a significant increase in the incidence of malignant lymphomas, Zymbal gland carcinomas, lung alveolar/bronchiolar adenomas and carcinomas, Harderian gland adenomas, preputial gland squamous cell carcinomas and female mammary gland carcinomas [30].

**Regulatory Measures**

Regulatory steps have actually been taken for benzene. The U.S. EPA has classified benzene as Category A; a known human carcinogen[31]. IARC concluded that benzene is a
Group 1 contaminant; carcinogenic to humans [32]. The NTP also identified benzene as a known human carcinogen in its Report on Carcinogens. These classifications are based on human evidence and supported by animal studies that benzene exposure has a causal relationship with cancer [26]. Due to its known human carcinogenic potential, the transport and emission levels, exposure limit, and maximum contaminant level of benzene have all been extensively regulated by many Agencies including the Department of Transportation, U.S. EPA, Food & Drug Administration and Occupational Safety and Health Administration. Benzene is considered a hazardous material and requires special labeling and handling. The U.S. EPA set the maximum contamination level (MCL) of benzene at 0.005mg/L in drinking water. OSHA’s permissible exposure limit of benzene in the work site is set at 1ppm over 8 hours [27].

**Tetrachloroethylene (PERC)**

PERC, a.k.a. perchloroethylene, is a nonflammable, halogenated alkene that is a colorless liquid at room temperature. PERC is a man-made VOC. It is primarily used as a cleaning solvent in dry cleaning, textile production and metal degreasing and as a chemical intermediate [33]. Structure, chemical and physical properties are shown below.

```
  Cl     Cl
 /      /  |
 Cl     Cl  Cl
```

Figure 1.2 Chemical structure of PERC. Obtained from a Google image search.
Table 1.2 Chemical and physical properties of PERC[33]

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>165.8 g/mol</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.6227 at 20°C/4°C</td>
</tr>
<tr>
<td>Melting point</td>
<td>-22.3°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>121.3°C</td>
</tr>
<tr>
<td>Log K&lt;sub&gt;ow&lt;/sub&gt;</td>
<td>3.4</td>
</tr>
<tr>
<td>Water solubility</td>
<td>206 mg/L at 25°C</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>18.5 mm Hg at 25°C</td>
</tr>
<tr>
<td>Vapor density relative to air</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*Exposure*

Humans are thought to be exposed to PERC through inhalation and ingestion of contaminated drinking water or food. Indoor air of buildings containing dry-cleaning services has higher concentrations of PERC compared to the indoor air from building without dry-cleaning services. Breath concentrations of PERC are significantly higher in dry cleaners and their family members (0.65ppm and 0.05ppm, respectively) compared to controls (0.001 ppm) [34]. Blood concentrations from the National Health and Nutrition Examination Survey (NHANES) participants ranged from below the limit of detection to ng/mL [34]. Higher blood levels have been found in occupationally exposed workers and populations with highly contaminated drinking water. Reported levels of PERC in breast milk have ranged from undetectable up to 43 μg/L in the general population [35]. PERC is released into the air primarily from manufacturing and processing facilities. The highest emissions come from the dry cleaning industry; however storing dry-cleaned garments can slightly raise the PERC levels in homes [33, 34, 36].

PERC degrades slowly and has an estimated half-life of 100 days in the air. PERC can also contaminate groundwater where it can persist for decades. Contaminations can occur
from improper disposal of chemicals from drying cleaning facilities or degreasing activities, which generate aqueous PERC waste. PERC biodegrades by reductive dechlorination and produces other potentially harmful chemicals, including TCE, trans-1,2-DCE and VC. PERC has been identified in at least 945 hazardous waste sites and detected in approximately 4% of U.S. aquifers. The median concentration in the aquifers was 0.09µg/L, and 0.7% of the samples exceeded the MCL for drinking water of 5µg/L[34].

Toxicity

Numerous studies have been conducted to assess the hazard and health risk of PERC exposure. Epidemiological and animal studies suggest the primary targets for PERC toxicity are the liver, kidney, nervous system and reproductive system. In humans, PERC has been associated with increased liver size, slight renal effects, decrements in nervous system function and spontaneous abortion. The limitation in most of the human studies is that humans are rarely exposed to just PERC. Other VOCs, such as the breakdown products of PERC, are often present. PERC is believed to directly affect the nervous system while metabolism to oxidative metabolites is thought to result in liver, renal and reproductive toxicities [34, 37].

PERC is potentially a human carcinogen. In human epidemiological studies with high quality exposure-assessment, PERC exposure was associated with bladder, multiple myeloma and non-Hodgkin’s lymphoma [37]. Other studies in humans suggest an association with esophageal, kidney, lung, liver, cervical, and breast cancer, but these studies are often inconsistent [34].

Regulatory Measures
The NTP determined that PERC is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals [33]. IARC concluded that PERC was probably carcinogenic to humans (Group 2A) based on limited evidence in humans and sufficient evidence in experimental animals [36]. The U.S. EPA also determined that PERC is likely to be a human carcinogen[37]. PERC is considered a hazardous material and is highly regulated. The MCL set by the EPA is 0.005 mg/L in drinking water and the permissible exposure limits set by OSHA is 100 ppm over 8 hours [33].

**Trichloroethylene (TCE)**

Trichloroethylene is a halogenated alkene and at room temperature is a colorless liquid, with moderate water solubility. Although it is a natural breakdown product of PERC, TCE is predominantly man-made to serve as an intermediate for hydrofluorocarbon production and as a solvent for metal degreasing [38]. Structure, chemical and physical properties are shown below.

![Chemical structure of TCE](image)

Figure 1.3 Chemical structure of TCE. Obtained from a Google image search.
Table 1.3 Chemical and physical properties of TCE[38]

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>131.4 g/mol</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.4642 at 20°C/4°C</td>
</tr>
<tr>
<td>Melting point</td>
<td>-84.7°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>87.2°C</td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>2.61</td>
</tr>
<tr>
<td>Water solubility</td>
<td>1.28 mg/L at 25°C</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>69 mm Hg at 25°C</td>
</tr>
<tr>
<td>Vapor density relative to air</td>
<td>4.53</td>
</tr>
</tbody>
</table>

*Exposure*

Inhalation and ingesting contaminated drinking water are the main routes of exposure to TCE in humans. TCE is a major ingredient in some household aerosols, adhesives, paint removers and degreasers (primarily for crafters and hobbyists). TCE has been found in food items, like avocados, and is likely due to contaminated water in food processing or degreasing of food processing equipment. TCE production has been declining over the last two decades and rural areas have lower levels than urban environments [36, 39, 40]. In the U.S., mean TCE concentrations have been determined for rural (0.03 ppb) and urban areas (0.460 ppb), and in areas near emission sources of TCE (1.2 ppb) [40]. In yearly monitoring of public water systems, TCE was detected in 4.9% of ground water samples and 14.8% of surface water samples. The median and maximum concentrations were 1.1 ppb and 159 ppb and 1.6 ppb and 50 ppb, for ground and surface water, respectively. According to NHANES, about 10% of the U.S. populations have detectable levels of TCE in their blood with a mean concentration of 0.017µg/L (ppb)[38].

Once TCE is released into the atmosphere it is degraded by reacting with hydroxyl radicals and has a half-life in air of about 7 days [40]. It is persistent in the environment due
to its constant release and potentially from formation as a breakdown product of the much more environmentally persistent PERC. TCE degrades much slower in water and will off-gas from surface water before degrading. Reductive dehalogenation by microorganisms can occur but at a very slow rate and TCE persists much longer in soil and underground water. TCE is the most frequently reported organic contaminant in groundwater. Industrial discharge of wastewater into streams is a major source of TCE contamination. TCE can also leach into the groundwater from landfills and has been identified in at least 1045 hazardous waste sites[40].

Toxicity

TCE poses a potential human health hazard to the central nervous system, kidney, liver, immune system, male reproductive system, and developing fetus. Human and animal studies have demonstrated that TCE exposure is associated with trigeminal nerve impairment (the nerve source for somatic sensation over the entire face, the eye, the nasal passages and the oral cavity) and vestibular system impairments (headaches, dizziness and nausea) [41]. TCE causes nephrotoxicity in the form of tubular toxicity. TCE exerts its actions predominantly through the metabolite S-dichlorovinyl-L-cysteine. TCE has been shown to cause some liver toxicity in animals, but there is only limited epidemiological evidence of TCE causing changes in liver function tests or liver toxicity [41].

Regulatory Measures

The NTP determined that TCE is reasonably anticipated to be a human carcinogen based on limited evidence of carcinogenicity from studies in humans and sufficient evidence from studies in experimental animals [38]. IARC determined that TCE is carcinogenic to
humans (Group 1) based on sufficient human and experimental animals studies [36]. EPA concluded TCE is carcinogenic to humans based on convincing evidence of a causal association between TCE exposure in humans and kidney cancer [41].

**trans-1,2-Dichloroethylene (trans-1,2-DCE)**

1,2-Dichloroethylene is also called 1,2-dichloroethene (DCE). It is a highly flammable, colorless liquid with a harsh odor. There are two forms of DCE; one form is cis-1,2-DCE and the other is trans-1,2-DCE. Sometimes both forms are present as a mixture. DCE is used most often as an ingredient of solvents and in chemical mixtures [42, 43]. Structure, chemical and physical properties of trans-1,2-DCE are shown below.

![Chemical structure of trans-1,2-DCE](image)

Figure 1.4 Chemical structure of trans-1,2-DCE. Obtained from a Google image search.

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>96.94 g/mol</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.2565 at 20°C/4°C</td>
</tr>
<tr>
<td>Melting point</td>
<td>-49.8°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>48.7°C</td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>2.06</td>
</tr>
<tr>
<td>Water solubility</td>
<td>4,520 mg/L at 25°C</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>331 mm Hg at 25°C</td>
</tr>
<tr>
<td>Vapor density relative to air</td>
<td>3.67</td>
</tr>
</tbody>
</table>
Exposure

The major route of exposure to the general population is through ingestion of contaminated drinking water. DCE may enter groundwater by leaching from contaminated waste disposal sites or more likely degradation of PERC or TCE. The general population in urban areas may also be exposed to low levels (0.013-0.076 ppb) in the air [43]. Sources of air-borne DCE are emissions from production companies, waste disposal sites and volatilization from contaminated waste waters.

DCE reacts with hydroxyl radicals in the air and the estimated atmospheric lifetime for trans-1,2-DCE is 5 days. In groundwater, DCE can slowly undergo further degradation to VC and potentially has a half-life of a couple months. Concentrations of DCE isomers detected in U.S. groundwater ranged from 0.25 to 0.28 ppb. Of the tested sources, DCE has been found at detectable concentrations in less than 5% of surface water sources and 21% of groundwater sources[43].

Toxicity

Very few studies have been conducted on DCE and human health concerns. There has been one documented death from excessive inhalation in the 1930’s, but no symptoms of toxicity were reported. Inhalation of trans-1,2-DCE at high concentrations is known to depress the central nervous system and cause nausea, drowsiness and fatigue. DCE is considered fairly nontoxic to animals. The LD₅₀ of trans-1,2-DCE is 7,900mg/kg in male Sprague Dawley rats when delivered via gavage [42]. Inhalation of trans-1,2-DCE at 200 ppm caused liver changes (fatty accumulation in lobules and Kupffer cells) and this study is the basis of the occupational inhalation MCL of 0.2 ppm [43]. There is no oral MCL for
trans-1,2-DCE and the numerous safety assessments have found that there is “inadequate information to assess the carcinogenic potential” of trans-1,2-DCE [45].

**Vinyl Chloride (VC)**

Vinyl chloride is a halogenated olefin compound that is very flammable and at room temperature is a colorless gas. Vinyl chloride is used by the plastics industry to produce polyvinyl chloride, which is used in many consumer and industrial products [46]. Structure, chemical and physical properties are shown below.

![Chemical structure of VC](image)

**Figure 1.5 Chemical structures of VC. Obtained from a Google image search.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>62.5 g/mol</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>0.9106 at 20°C/4°C</td>
</tr>
<tr>
<td>Melting point</td>
<td>-153.7°C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>13.3°C</td>
</tr>
<tr>
<td>Log Kow</td>
<td>1.62</td>
</tr>
<tr>
<td>Water solubility</td>
<td>8.8 mg/L at 25°C</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>2980 mm Hg at 25°C</td>
</tr>
<tr>
<td>Vapor density relative to air</td>
<td>2.15</td>
</tr>
</tbody>
</table>
Exposure

The major route of exposure to the general population is inhalation and ingestion of contaminated drinking water. Smoking may also expose individuals to VC. Unless living near emission sources, the general population is rarely exposed to VC. Ambient air does not typically contain detectable levels of VC, however near emission sources levels can range from trace to 2,600µg/m$^3$[48]. VC is rarely detected in public drinking water; the U.S. EPA estimates that 0.9% of the U.S. population is exposed to 1 µg/L or higher VC and only 0.3% of the population is exposed to higher than 5 µg/L. Occupational exposure is the most common way the population is exposed to VC [48-50].

VC is primarily a man-made substance, but can naturally be formed from the breakdown of PERC or TCE. VC is mostly released from VC and PVC manufacturing plants but can leach into groundwater from spills and landfills. VC in the atmosphere is removed by reacting with hydroxyl radicals and has a very short half-life of a few hours. The half-life of VC in groundwater is much longer than in the air. There are certain microorganisms and chemical reactions that can degrade VC however; volatilization is the main route of transport out of water. VC has been identified in at least 622 hazardous waste sites [48].

Toxicity

VC is known to have toxic effects on several organ systems, with liver being the most sensitive in animal studies. High doses, such as those experienced in occupational settings, can cause neurological effects, such as headaches, dizziness, loss of consciousness and Raynaud’s phenomenon (numbness in fingers and cold sensitivity) [48]. VC is associated with liver impairment and damage and is strongly associated with liver angiosarcoma in
Angiosarcoma is a neoplasm arising from vascular endothelial cells in the liver, and is rare in humans. Other tumors may be associated with VC such as, liver cancers, brain soft tissue and nervous system cancer. VC metabolites, chloroethylene epoxide and chloroacetaldehyde, are both genotoxic and can form DNA and protein adducts [48].

**Regulatory Measures**

Animal studies have confirmed the carcinogenicity of VC in several rodent species and via different routes of exposure. Liver, mammary, skin, lung and Zymbal’s gland tumors have all been associated with VC exposure in the rat [48]. The U.S. EPA has classified VC as Category A, a known human carcinogen [49]. IARC classified VC as Group 1; carcinogenic to humans [50]. The NTP also determined that VC is a known human carcinogen [46]. These classifications are based on human evidence and supported by animal studies that VC exposure has a causal relationship with cancer.

Men and women were exposed to VOCs at Camp Lejeune in a manner that was not recapitulated in the 2 year bioassays of PERC, TCE, VC and benzene. Rats and mice were exposed to high concentrations of VOCs as adult animals and to a single compound, via inhalation or oral gavage. Marines and the children of the Marines stationed at Camp Lejeune were exposed to a mixture of VOC at a low concentration, rather than a single compound. Children of Marines could have been exposed to VOCs in utero, as young children and/or during puberty and the Marines themselves could have been as young as 17 when stationed there. In addition, VOC exposure likely occurred by both ingestion and inhalation. Any three of these differences in VOC exposure could have made the mammary gland a sensitive developing target organ and led to an increased risk of developing breast
This dissertation will address the first two key differences in exposure to VOCs and will discuss the limitations and future directions with inhalation and oral exposure to VOCs. My hypothesis is the following: human relevant drinking water exposure to VOCs, during critical periods of mammary gland development, will alter mammary gland development and increase susceptibility to DMBA-induced tumor formation in male and female HSD rats. Before evaluating the effects of VOCs on the mammary gland, Chapter 2 will define both normal and chemically altered male and female HSD rat mammary gland development.
REFERENCES


35. EPA, U.S., *Sources, emission and exposure for tetrachloroethylene (TCE) and related chemicals* 2001, U.S. Environmental Protection Agency Washington, DC


CHAPTER 2: An Atlas of Mammary Gland Development in Male and Female Harlan Sprague Dawley Rats: Examples of Accelerated and Delayed Development

Introduction

The mammary gland is a complex tissue made of multiple cellular compartments: the epithelium and the surrounding stroma, which includes fibroblasts, adipocytes, and immune cells [1]. Mammary gland development is orchestrated by many different hormones and growth factors [2]. Rapid mammary gland development occurs during embryogenesis, puberty, pregnancy and lactation. Unlike most organs, the majority of mammary gland development occurs after birth. Although there are specific differences in timing, female human and rodent mammary glands undergo a similar developmental process [3, 4]. During embryogenesis, the mammary bud and the resulting primary duct extend into the developing mammary fat pad. Secondary side branching of the primary duct occurs and a small ductal tree is present at the time of birth. Prior to puberty, the gland undergoes isometric growth and as puberty progresses, the ductal growth occurs exponentially. In adulthood, the mammary gland enters a resting static state with slight hormone-induced changes in the female related to the estrous cycle [5]. At this point, the overall structure of the adult female mammary gland is tubuloalveolar, meaning that the gland is composed primarily of ducts with lumens. Starting at pregnancy and continuing through lactation, the overall structure of the mammary

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1This chapter has been submitted as an article in the Journal of Toxicologic Pathology by the authors: Filgo, A.J., Foley, J.F., Borde, A.R., Reed, C.E., Chappell, V.A., Alexander, L.B., Borde, P.R., Hayes, S.A., and Fenton, S.E.
gland changes and becomes lobuloalveolar, where alveolar lobules, surrounded by the myoepithelium, are the predominant structures serving the function of milk production.

Improper mammary gland development has very important health consequences at different life stages. There is accumulating evidence in rodents that fetal exposure to certain chemicals may lead to inappropriate development of the mammary gland in offspring, which may lead to nipple/areola retention in males and enhanced or delayed ductal development in females [6]. During puberty, endocrine disruption can result in early mammary gland development, a common indicator of precocious puberty in girls, or gynecomastia, which is abnormal breast development in males. Both of these are associated with an increase in the risk of certain cancers developing later in life. Abnormal mammary gland development during lactation can lead to an inadequate production of milk.

The most important potential disease outcome involving the mammary gland in humans is breast cancer. According to the American Cancer Society, in the United States, breast cancer develops in about 1 in 8 women and 1 in 1,000 men [7]. In women, breast cancer is the most commonly diagnosed form of cancer, and breast cancer breast cancer kills more women than any other type of reproductive cancer or endocrine-related cancer in the U.S. [8, 9]. Approximately 85% of breast cancers occur in women who have no family history of breast cancer [3].

Rodent studies have proven that the mammary gland is a sensitive tissue to the effects of EDCs, which are exogenous chemicals that, individually or in mixtures, may interfere with any aspect of hormone action [10]. There are certain stages of development in which the mammary gland is more susceptible to the effects of EDCs. These “windows of
susceptibility” for the mammary gland are during embryogenesis, puberty and pregnancy/lactation; when the mammary gland is undergoing extensive proliferation. Terminal end buds (TEBs) are highly proliferative epithelial structures present during puberty in the rodent mammary gland. Since TEBs are the precursors of future branching ducts, damage or altered programming of these mammary gland structures can have lasting consequences within the structure of the mammary gland [6]. EDCs have been reported to change mammary gland development following prenatal, neonatal, and peripubertal exposure (reviewed in [6]). Chemical disruption of mammary gland development during the early windows of susceptibility (prenatal) can result in impaired lactation and altered susceptibility to cancer as adults.

In addition to the developmental similarities, the cellular composition of human and rodent mammary glands is also similar. In humans, the functional portion of the mammary gland is called the terminal ductal lobular unit (TDLU) and in rodent species is it called the lobuloalveolar unit (LAU) or the terminal ends, not to be confused with TEBs. Both the TDLU and LAU are composed of similar luminal cuboidal epithelium and surrounding myoepithelial cells, with a tubuloalveolar morphology (abundant ducts and few alveoli) [11-12]. During pregnancy and lactation, the mammary epithelium shifts to a lobuloalveolar morphology, with abundant alveolar lobules.

The structure of mammary glands in men is considered a rudimentary form of the female gland [13]. On the other hand, mammary glands of rodents exhibit several sex-associated structural differences. Male rats have branched mammary epithelium present at birth, though they have been reported to be smaller in size than those of females, and the mammary epithelium of sexually mature males have a permanent lobuloalveolar structure.
Additionally, male mice may occasionally possess a residual stalk of epithelial tissue that is structurally similar to the female primary duct [14], and recent data suggests these stalks may respond to EDC exposure [15].

The rat mammary gland is an excellent model to study the effect of environmental chemicals that may impact breast cancer risk in humans [16]. Altered rodent mammary gland development during windows of susceptibility can be evaluated through the use of mammary gland whole mounts. Whole preparations of the mammary gland are fixed, defatted, stained and coverslipped with permount on a charged microscope slide. The glandular epithelial and stromal cells are stained with carmine and the outlines of adipocytes are intact. Whole mounts allow for evaluation of the total number and relative abundance of mammary gland ductal structures, including TEBs, overall growth of the epithelial cells, and branching density. [17].

The current studies were performed to generate a pictorial atlas that characterizes mammary gland development in male and female Harland Sprague Dawley (HSD) rats. The study focuses on HSD rats as this strain is currently the rat of choice in some NTP test guideline studies. Embryonic bud development was characterized histologically using fetal transverse hematoxylin and eosin (H&E)-stained sections starting on E15.5 and continuing through to E21.5. Postnatal development was evaluated using mammary gland whole mounts, coronal histology sections, and sex hormone receptor immunohistochemically (IHC)-stained sections prepared from the fourth and fifth inguinal gland starting at postnatal day (PND) 1 and continuing through to PND 70. To provide representative examples of accelerated or delayed mammary gland growth and differentiation in males and females, diethylstilbestrol (DES) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (respectively) was
administered to a subset of pregnant dams during embryonic mammary gland organogenesis. This mammary gland atlas addresses male- and female-associated differences in both normal and chemically altered mammary development of HSD rats. The use of this atlas will benefit future studies assessing morphological changes in mammary gland endpoints in the HSD rat and other related strains.
Materials and Methods

Animals

Three blocks of timed-pregnant Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). Pregnant dams arrived on GD 10 or 13 and were housed individually in polypropylene cages with bedding and nestlets and switched to a rodent phytoestrogen-reduced diet (Zeigler; Madison, WI). Water and chow were supplied ad libitum. The animals were maintained in a room with a 12:12-hour light/dark cycle, at 20–24 °C, and 40–50% relative humidity. All animals were treated humanely and with regard for alleviation of potential suffering, as approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

Atlas Experiment

Block One

Timed-pregnant dams were randomly distributed among three treatment groups (n=9/group) with mean body weight (BW) being equivalent between the groups. Dams in the three groups were treated by oral gavage on GD15 and GD18 with either 5 ml sesame oil/kg BW (vehicle control), TCDD (0.5 µg TCDD/kg BW), or DES (10 µg DES/kg BW). Dam BW was recorded daily throughout gestation. TCDD was purchased from Radian Corporation (Austin, Texas), and DES was purchased from Sigma (St. Louis, Missouri). Dosing solutions were prepared fresh on the days of treatment. The dose of DES was based on previous studies [18, 19] demonstrating no effects on prenatal loss or delayed parturition. The TCDD dose was derived from previous studies to avoid changing maternal weight gain,
but to give a dose to fetuses that may affect mammary development [17]. Treated and control dams were allowed to give birth and neonates were equalized to 10 pups per litter (5 male, 5 female) on PND 4 to equalize growth potential across litters, minimizing litter effects. On PNDs 1, 4, 8, 12, 15, and 21, one dam and litter per treatment group were euthanized and mammary glands were collected from the dam and all pups for whole mounts and histology. At PND 21, all remaining litters were weaned. Offspring (3 males and 3 females per litter) were sacrificed on PNDs 33, 46, and 70 to continue to monitor post-pubertal mammary gland development.

**Block Two**

Untreated timed-pregnant dams (n=14) were utilized for fetal mammary gland development. Two dams were sacrificed and male and female fetuses were collected (n=3 fetuses/sex collected) for mammary gland evaluation on E15.5, 16.5, 17.5, 18.5, 19.5, 20.5 and 21.5.

**Block Three**

Untreated timed-pregnant dams (n=8) were utilized for fetal mammary gland development. Two dams were sacrificed and male and female fetuses were collected (n=3 fetuses/sex collected) for mammary gland evaluation on E18.5, 19.5, 20.5 and 21.5.

**Tissue Samples**

The fourth and fifth inguinal mammary glands are the easiest and largest glands to assess and were removed from both sides of the body, without the skin, in all dams and PND 1- PND 70 pups from block one. One side was collected for whole mount and the other for
H&E or IHC. The samples for whole mount were fixed in Carnoy’s solution and stained in Carmine Alum as described in [17]. A male and female representative mammary gland was selected from each age group for pictorial representation.

The fourth (and often fifth) gland on the contralateral side from the whole mounted gland was fixed in 10% neutral buffered formalin for 24 hours and transferred to 70% ethanol. Tissue samples were trimmed, processed and embedded in paraffin. Each block was step-sectioned until the lymph node was visible. After reaching the lymph node, four serial 5 µm sections were collected for H&E and IHC staining. The following biomarkers were assessed by IHC staining: estrogen receptor alpha (ERα), progesterone receptor (PR), and androgen receptor (AR).

Fetuses in blocks two and three were euthanized with wet ice and decapitation (E15.5-E 17.5) or by decapitation (E18.5-E21.5). In block two (n=14), fetuses were fixed in Bouin’s Fixative (Poly Scientific R&D Corp. Bay Shore, NY) for 48 hours. In block three (n=8), Bouin’s Fixative was injected into the abdominal cavity for better fixation and fetuses were further fixed in Bouin’s Fixative for 48 hours. Following fixation, a transverse cut was made at the level of the umbilicus. The upper half of the embryos was discarded while the bottom half was rinsed three times in 70% ethanol to remove the Bouin’s solution and placed in phosphate buffered saline at 4 °C. Samples were then processed and embedded in paraffin. For optimal evaluation of the fetal mammary glands (E15.5- E21.5), the fetus was embedded in the transverse plane. The fetus was exhaustively sectioned (6 µm) through, in the region of the 4/5th mammary gland. Fetal cross-sections that potentially contained mammary tissue were identified by light microscopy, then H&E stained.
**ERα and PR immunohistochemistry**

Tissue sections were deparaffinized and rehydrated through a series of alcohols to phosphate buffered saline. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Heat induced antigen retrieval was performed using citrate buffer pH 6.0 (Biocare Medical, Concord, CA) at 120 °C for 5 minutes in a decloaking chamber (Biocare Medical). The sections were incubated with 10% normal horse serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 20 minutes. Endogenous biotin was quenched with the Avidin-Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). Sections were incubated with either anti-estrogen receptor alpha antibody at a 1:50 dilution (Beckman Coulter, Brea, CA; catalog # IM1545) or anti-progesterone receptor antibody at a 1:150 dilution for 1 hour (Beckman Coulter, Brea, CA; catalog # IM1546). For a negative control, sections were incubated with mouse IgG1 isotype control serum in place of the primary antibody (BD Biosciences, San Diego, CA. Sections were incubated with the secondary antibody, horse anti-mouse secondary antibody (Vector Laboratories) for 30 minutes at 1:1000 dilution and then labeled (Vector R.T.U. Vectastain Kit, Vector Laboratories) for 30 minutes. The antigen-antibody complex was visualized following a 6-minute incubation with 3, 3’ diaminobenzidine (Dako Corporation, Carpinteria, CA). The sections were counterstained with modified Harris hematoxylin, dehydrated, cleared and coverslipped with Permount.

**AR immunohistochemistry**

Slides for AR IHC underwent deparaffinization, HIER and were blocked for endogenous peroxidase as described above. The sections were incubated with 10% normal
donkey serum for 20 minutes, followed by the Avidin-Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) to quench endogenous biotin. Rabbit polyclonal anti-androgen receptor antibody (Santa Cruz Biotechnology, Dallas, TX; catalog # sc-816) was applied to the sections for staining at a dilution of 1:100 for 1 hour. Negative control slides received rabbit IgG serum at the same concentration as the primary antibody. A donkey anti-rabbit biotin conjugated secondary antibody was applied for 30 minutes at a 1:500 dilution. Labeling, chromogen incubation, counterstaining and coverslipping were identical to the ERα and PR protocol described above.

**ERα/PR/AR quantification**

A modified Quick Score method [20] was used to report IHC staining for ERα, PR and AR in the mammary tissue. The overall intensity of the staining is scored on a scale of 0-3; where 0= no staining/negative; 1= weak staining; 2= intermediate staining; 3=strong staining. The estimated percentage of cells that are stained throughout the entire gland are scored on a scale of 1-6; where 1= 0-4% staining; 2= 5-19% staining; 3= 20-39% staining; 4= 40-59% staining; 5= 60-79% staining; 6= 80-100% staining. The Quick Score is then calculated by multiplying the average intensity (0-3) by percent of cells stained (1-6). Quick scores range from 0-18 and only used for nuclear localized proteins. Nuclear staining was quantified by two reviewers unaware of the sample age, sex, or treatment group.

**Brightfield Slide Scanning**

Brightfield digital images were taken from slides scanned on an Aperio ScanScope XT™ instrument (Vista, CA) using ImageScope™ software (v9.0, Aperio). The extraction feature in Image-Scope™ was used if a digitally scanned image required rotation. Image
enhancement (imaging, resizing and white balance correction) was done using Adobe Photoshop CC (Adobe Systems Incorporated, San Jose, CA, USA).
Results

Harlan Sprague Dawley Rat Mammary Gland Atlas

_H&E fetal mammary bud development_

Sections of E15.5-21.5 fetuses from untreated dams demonstrate the progression of male and female mammary gland bud development (Fig. 2.1, 2.2, 2.3 and 2.4). At E15.5, the early mammary bud consists of a sphere of concentrically arranged epithelial cells subjacent to the plane of the surrounding epidermis (Fig. 2.1A-B and Fig. 2.3A-B). Cells surrounding the bud continue to elongate, condense and later connect to the skin surface by a stalk of epidermal-like cells at E16.5. During this gestational time point, the mature mammary bud surrounded by three to five layers of condensed mesenchyme, is referred to as the primary mammary mesenchyme. Developing from the mesenchyme, along with the formation of the ectodermally derived mammary buds, are the support structures including the adipose tissue, which later forms the fat pad containing blood vessels, lymphatics and connective tissue (Fig. 2.1C-D and Fig. 2.3C-D). The bud remains quiescent from E16.5 to E17.5 and is relatively unchanged except for further progression into the underlying adipose tissue. At E18.5 in the male mammary gland, under the influence of androgens (testosterone) [21], the epithelial bud becomes separated from the epidermis as a result of apoptosis of the underlying mammary mesenchyme (Fig. 2.4). In the female, the mammary gland begins to invaginate where the nipple will develop (Fig. 2.2A-B). Invagination continues in the female until E19.5 when the outer structure of the nipple forms (Fig. 2.2C-D). At E20.5, the nipple sheath forms in the female rat (Fig. 2.2E-F). This is a modification of the skin present in the immediate vicinity of the mammary duct. The epidermis thickens and projects down into the dermis in an umbrella-like fashion, forming a ridge that surrounds the origin of the primary epithelial
duct. In contrast, the male mammary gland does not invaginate or undergo nipple formation.

At E21.5, solid epidermal cords extend from the mammary bud and grow through the mammary mesenchyme into the secondary mammary mesenchyme, or fat pad precursor tissue, to form mammary sprouts in both the male and female (Fig. 2.2 G-H and Fig. 2.4 G-H). Once the mammary sprout has reached the fat pad, it begins a process of ductal branching morphogenesis that gives rise to the rudimentary ductal tree, consisting of a primary duct and 15-20 secondary branches, which is present at birth.

**Cellular morphology in pups and pregnant/lactating dam**

Cellular morphology of the female and male mammary gland can be compared/contrasted using H&E-stained longitudinal sections (Fig. 2.5 and 2.6, respectively). Although mammary gland development begins during gestation, the majority of development occurs after birth. Although samples were collected at seven ages, four age groups are presented in Fig. 2.5 and 2.6. These ages are often used for mammary gland comparisons in a variety of research studies; PND 4 (culls from litter size equilibration), PND 21 (extra weanlings), PND 46 (about the age chemical carcinogen is delivered), and PND 70 (young adult, similar to PND 90). At PND 4, both females and males displayed a simple mammary gland primarily composed of branching ducts and a few buds (Fig. 2.5A and 2.6A). Histologically, the ducts of the mammary gland of the pre-pubertal female and male rat are characterized by a single or double layer of cuboidal epithelium, which after branching a number of times and causing extension toward the lymph node, becomes multilayered to form the TEBs. These similarities in cellular structure continued through weaning (Fig. 2.5D and 2.6D). Around PND 21, the terminal end buds begin to cleave into clusters of three to five alveolar buds each with a centrally located lumen surrounded by a
layer of cuboidal epithelial cells. Between PND 21 and PND 46, the females will have undergone vaginal opening, begun regular hormone surges, and have an established estrous cycle [22]. The males are peri-pubertal at PND 46, likely progressing through preputial separation a few days prior to this timepoint [23]. By PND 46, there was an increase in the lobular architecture of the male gland. Luminal epithelial cells contained enlarged vesicles within the lumens and by PND 70, the male mammary gland is a sexually mature gland. The glandular tissue is greater in volume and is organized into lobules of alveoli that consist of large, pale staining, foamy and vacuolated cells that are arranged around indistinct lumen that occasionally contain small amounts of eosinophilic secretory material. Ducts are lined by multiple layers of epithelium (pseudostratified or striated epithelium) and also have indistinct lumen and resemble alveoli. The mammary structure in the male is termed lobuloalveolar (Fig. 2.6J). At PND 70, the mammary gland of the mature, virgin female rat varies little histologically from that of a prepubertal female except for more abundant branching, narrower ducts, and more numerous alveolar buds and lobules. The female gland at this time point is characterized by branching tubular ducts, terminal end buds and alveolar buds, and clusters of patent alveoli are now tubuloaveolar structures (Fig. 2.5J).

Mammary epithelium from DES-exposed females is similar in architectural appearance when compared to the controls at all time-points in H&E-stained sections. A tubuloaveolar structure is present with no appreciable difference in cellular structure or organization (Fig. 2.5B, E, H, and K). Mammary tissue from TCDD-exposed females contained significantly fewer terminal branches and alveoli when compared to controls and DES-exposed animals (Fig. 2.5C, F, I, and L). The TCDD induced delay of mammary gland development was persistent over time and was apparent at PND 21 and PND 46. However,
by PND 70 the mammary epithelium of TCDD-exposed females was more similar to age matched controls and DES-exposed females.

The mammary epithelium of DES-exposed and control males were indistinguishable from each other from PND 4-46 (Fig. 2.6B, E, and H). At PND 70, when the control mammary glands became sexually mature, mammary tissue from DES-exposed animals contained typical lobuloalveolar structures centrally, matching the appearance of the control; however, the outer edges of the mammary gland maintained a tubuloalveolar structures characterized by a predominance of ducts and fewer alveoli (Fig. 2.6K). These findings may suggest that the DES-exposed males were feminized, or more likely, the mammary epithelium exhibited a delay in maturation. Similar to females, the mammary glands of TCDD-exposed males contained significantly fewer terminal branches and alveoli when compared to controls and DES-exposed animals (Fig.2.6C, F, and I). The TCDD induced delay of mammary gland development was persistent over time and was apparent at PND 4-46. At PND 70, the mammary epithelium of TCDD-exposed males was more similar to age matched controls males (Fig. 2.6L).

Sections of H&E-stained mammary glands from pregnant and lactating control dams are shown in Fig. 2.7. At GD 15, the mammary gland already had acquired a lobuloalveolar structure (large, vacuolated cytoplasm with a diminished lumen) with abundant adipose tissue (Fig. 2.7A). From GD 15 through GD 21, extensive ductal growth, lobular development, and alveolar maturation occurs to form milk-producing glands. With growth of the gland, there was a concurrent reduction in the amount of adipose tissue. (Fig.2.7B-C). The secretory alveoli became lined by low cuboidal epithelium, surrounded by a layer of myoepithelial cells, a basement membrane and a network of capillaries and lymphatics. The
continued growth of the mammary gland during pregnancy was due to increases in the height of the epithelial cells and expansion of the lumen of the alveoli. Lactation begins after the dam gives birth. Alveolar lumens were markedly expanded and distended with milk by PND 4 (Fig. 2.7D). Since the mammary epithelial growth is quite extensive from late pregnancy to early lactation, it is difficult to distinguish ducts and adipose tissue on the mammary gland whole mounts due to the dramatic uptake of stain by the LA epithelium (data now shown). H&E-stained mammary sections are the best way to evaluate the late pregnant and lactating rat mammary gland.

**Differences in male and female ER\(\alpha\), PR and AR stained sections**

Nuclear ER\(\alpha\) and PR in control male and female HSD rat mammary epithelia were not detectable at birth. In females at PND 8, no nuclear ER\(\alpha\) staining was detected. However, faint cytoplasmic staining was present, which increased in amount and intensity at PND 33 (Fig. 2.8A and B). At PND 70, the cytoplasmic staining was diminished (Fig. 2.8C). ER\(\alpha\) was detected in epithelial nuclei as early as PND 21 in control females. Quick Score analysis revealed no significant difference in the amount of ER\(\alpha\) positive nuclei in the mammary epithelium as the control animals progressed from weaning (PND 21) through adolescence (PND 33) to adulthood (PND 70). ER\(\alpha\) positive nuclei were observed in mammary glands of DES-exposed females on PND 15, while expression was not detected in control mammary glands until PND 21. Some TCDD-exposed animals expressed ER\(\alpha\) at PND 21 (Fig. 2.10A). ER\(\alpha\) was observed for the first time in mammary glands of the control, DES-exposed, and TCDD-exposed males at PND 33. After PND 33, ER\(\alpha\) in male mammary glands was very low, if present at all (Fig. 2.9A-C and Fig. 2.10B).
PR in the nuclei of control female mammary epithelium was first detected at PND 33 and continued through PND 70. PR in mammary epithelium of DES-exposed female rats was first observed at PND 33, but was delayed in appearance in mammary epithelium of TCDD-exposed females until PND 46 (Fig. 2.10E). PR was not present in male mammary epithelium at any time point or under any treatment condition (Fig. 2.9D-F).

Androgen receptor (AR) positively stained the nuclei in control male mammary epithelium as early as PND 4. Presence of AR was variable but continued to increase in both staining intensity and number of positively stained cells until about PND 46. By PND 70, AR nuclear receptor staining diminished to low levels in control males. While maximum AR stained mammary epithelium was observed at PND 45 in all males, the Quick Score was lower in glands of DES-exposed males compared to those of other males. Mammary glands of control males consistently exhibited slightly higher Quick Scores and more consistent AR stained mammary epithelium than glands of chemically exposed males (Fig. 2.10C and D). In females, positive nuclear AR stained mammary epithelium was variable and staining was very low throughout development when present (Fig. 2.8G-I and Fig. 2.9G-I). The Quick Score results are summarized over time, by sex, in Fig. 2.10.

Whole mounts analysis of normal and chemically altered mammary gland development

Normal and altered mammary gland development in both females and males was demonstrated with whole mounts (Fig. 2.11, 2.12 and Fig. 2.13, 2.14, respectively). At PND 1, the mammary gland of an unexposed male and female HSD rat is composed of a primary duct and several branched secondary ducts. Mammary epithelium of the fourth gland had extended longitudinally to the lymph node and there were few alveolar buds present at birth
From about PND 4 to PND 21, alveolar buds increase in number until they have densely covered almost all the secondary ducts. Longitudinal epithelial growth has not extended past the lymph nodes and very few TEBs have formed. TEBs are the highly proliferative terminal ductal structures primarily responsible for epithelial extension through the fat pad. Between PND 21 and PND 33, there is a dramatic increase in the number of TEBs in both males and females. Longitudinal growth is rapid and the epithelium has grown past the lymph nodes; lateral growth has also increased and alveolar buds begin to form tertiary ducts (Fig. 2.12A and Fig. 2.14A). Also by PND 33, the fourth and fifth glands may have grown together (Fig. 2.12A and Fig. 2.14A). As puberty begins in the female around PND 34 [22], mammary epithelium proliferation accelerates quickly and by PND 46 and PND 70, growth continues to extend to the outer edges of the fat pad, TEBs have differentiated into terminal ends and the alveolar buds within the gland have become mature alveolar structures (Fig. 2.12D, G).

Mammary glands developed similarly in both sexes before puberty. However, an unusual phenomenon was observed in males in that the fifth gland was not always present in this rat strain. The fifth mammary gland was missing in 50% of males (data not shown). Because of this phenomenon, only the fourth gland was evaluated in the male rats. Differences between the fourth mammary gland in male and female rats were not apparent until after PND 21 (Fig. 2.11A, D, G and Fig. 2.13A, D, G). After PND 21, the female mammary gland developed at a much faster and more uniform rate compared to those of males. By PND 33 in females, the mammary epithelium had grown past the lymph node, the fourth and fifth glands had grown together, and there was lateral growth to the edge of the fat pad. The number of TEBs had reached a maximum and terminal ends developed where the
The gland had reached the edge of the fat pad. Compared to female glands, the male glands had reduced longitudinal and lateral growth and fewer TEBs. (Fig. 2.12A and Fig. 2.14A). There was a greater degree of variability of mammary gland development among the male mammary glands after PND 21. Of the animals that had a fifth gland, not all had grown together at PND 33. These factors made selecting a representative image much more difficult. The epithelial area of male mammary glands changed little after PND 33, and in most glands did not ever reach the edges of the fat pad. In female mammary glands, TEBs begin to diminish as the gland reaches the end of the fat pad at PND 46, while in the males; TEBs differentiate into terminal ends prior to reaching the end of the fat pad (Fig. 2.12D and Fig. 2.14D). By PND 70, very few TEBs were found in the female mammary gland. (Fig. 2.12G). The male mammary gland became very dense and exhibited the appearance of a sexually mature male mammary gland by PND 70, with prominent alveolar sacs and ducts that were barely visible (Fig. 2.14G).

Comparing whole mount mammary glands from the DES-exposed females to the controls, the prepubescent females (PND 8-21) had dense and more abundant alveolar budding on the ducts (Fig. 2.11B, E, and H). TCDD-exposed females had very stunted mammary glands compared to controls. From PND 1-PND 21, the mammary glands of TCDD-exposed females exhibited consistently less branching and little alveolar budding off the ducts (Fig. 2.11C, F, and I). This is especially apparent in Fig. 2.11I. The fourth and fifth glands of both control and DES-exposed females had grown together by PND 33, while those of TCDD-exposed females had not (Fig. 2.12B-D). Despite having not grown together, there were still fewer TEBs present in the glands of TCDD-exposed females. The growth of the mammary epithelium in TCDD-exposed animals never caught up to that of the control
glands. By PND 70, mammary glands of TCDD-exposed females had not reached the end of the fat pad and there were more TEBs present than in controls (Fig. 2.12I). Differences between the treatment groups were more evident when compared by mammary whole mount vs H&E-stained section, likely because there was more information present on the slide to use in the tissue assessment.

There was no marked difference in mammary gland size, branching/budding density or TEBs formation between the controls and DES-exposed male animals until PND 70, where the mammary epithelium typically continued to grow further through the fat pad than the mammary epithelium of the controls or TCDD-exposed males. The centers of the glands were dense, due to sexual maturation and conversion to a lubuloalveolar epithelial structure, but the outer edges were less dense, like a female or immature gland, similar to what was found in the H&E stained glands (Fig. 2.13B, E, and H, and Fig. 2.14B, E, and H). The mammary epithelium of TCDD-exposed males was underdeveloped compared to the controls. In the prepubescent males (PND 1-21), there was less budding off the ducts compared to the controls (Fig. 2.13C, F, and I). The longitudinal and lateral growth of the mammary epithelium of the TCDD-exposed males caught up to the controls by PND 33 (Fig. 2.14C). Further mammary epithelium expansion was minimal and the whole mounts of the TCDD-exposed males appeared similar to the controls in terms of size and density through to PND 70 (Fig. 2.14F- I).
Compiled literary findings on the progression of mammary gland development and hormones levels in female rats.

Fig. 2.15 is a compiled literary search on what is currently known about female rat mammary gland development, receptors present in the mammary gland and sex hormone levels throughout the life span (mostly in Charles River Sprague Dawley rat). Data from our studies has been added to the figure (the Developmental Effects and Receptor Expression for ERα, PR and AR) to fill in data gaps.
Summary

The NTP has moved away from the use of the inbred Fisher 344/N rat due to decreased fecundity, sporadic seizures and high rates of mononuclear cell leukemia and testicular neoplasia [24]. One of the preferred testing strains is now the HSD rat because the litter size, sex ratio, and body weights were favorable [25]. Guideline studies (modified one generation reproduction studies, reproductive assessment by continuous breeding studies and 2-year bioassays) have been modified to include assessing mammary gland development in their standard testing protocols [26]. Since the majority of current studies in the literature are based on the Charles River Sprague Dawley strain and strain differences in mammary gland development have not been documented, it was important to make an atlas of normal HSD rat mammary gland development. Additionally, a comparison of normal and chemically altered mammary glands would fill data gaps in HSD mammary gland development. This atlas compares the growth and timing of critical mammary gland events starting at mammary gland bud formation and continuing through sexual maturity. We confirmed that the female mammary gland undergoes similar stages of development as has been previously shown in other rat strains [16, 27-29] and that the male mammary gland is structurally and morphologically similar to that of the females up until the onset of puberty. The HSD female mammary epithelium proliferates and spreads through the fat pad much faster compared to other strains (Stanko J, personal communication, January 22, 2015). By PND 33, 100% of the fourth and fifth mammary glands in the control females had already grown together (data not shown). This event does not occur until around PND 45 in other strains, such as Charles River Sprague Dawley (Stanko J, personal communication, January 22, 2015) and Long Evans rats [27]. This information is vital for future carcinogen-induced mammary tumor
model studies in rats, as the HSD female rat may have a smaller window of susceptibility to mammary carcinogens, compared to other rat strains. As a result, the HSD rat may be less susceptible to EDCs effects on mammary gland development than other strains.

The adult female rat mammary gland has been extensively studied but developmental time points are less understood. The atlas developed within these studies, adds or confirms important information on the development and timing of the mammary glands of male and female HSD rats. The female mammary gland that is undergoing lactation or involution has been well documented (Fig. 2.7), but embryogenesis and puberty are other critical windows of susceptibility that deserve more attention. The developmental events in Fig. 2.15 were demonstrated in the female HSD rat in the atlas. The mammary gland bud developed by E15.5 and epithelial ducts with a lumen were present at birth and continued to rapidly expand through to adulthood. TEBs began to form just before puberty and differentiated into mature lobules once reaching the end of the fat pad or other mammary glands at the beginning of adulthood. Chemically exposed HSD female rats showed expected and unexpected results. The TCDD model of stunted mammary gland growth in rats showed expected results [17]. The mammary gland was smaller and developmentally delayed compared to the control starting at PND 8 and never fully recovered by PND 70. The model for accelerated growth, with DES did not result in noticeably accelerated mammary gland development in the female. However, feminization may have occurred in the males exposed to DES in utero. This strain of rat may not be as sensitive to DES treatment as other strains, especially when considering that the HSD already exhibits accelerated mammary gland development compared to other strains (Stanko J, personal communication, January 22, 2015). During windows of susceptibility, hormone disruption, changes in receptor population in mammary
glands or altered mammary gland development can result in later life effects, including impaired lactation and breast cancer development [6]. Since the mammary gland is such a dynamic organ and is sensitive to various perturbations, it is critical to know both what is normal and what changes early in life will result in adverse outcomes later in life.

The steroid hormone receptors, estrogen and progesterone, have also been extensively studied in the adult mammary gland [30-33] and their presence or absence is shown in Fig. 2.15. The androgen and protein receptors were less well defined in the mammary gland and there are currently data gaps in the neonatal and peripubertal time points for some of these receptors. The ERα, PR and AR staining performed in these studies added information to Fig. 2.15. The findings in this study agree with previously published ERα data [34] in female Sprague Dawley rats in that no nuclear staining was observed until PND 21 in the controls. PR was not found in neonatal females but as previously reported in other strains [35] it is present in prepubertal and adult females. AR staining data added new information to the table, demonstrating that AR was not present in mammary epithelium nuclei until PND 15 in female controls, and only very low expression through PND 70. DES-exposed animals were the only group to have nuclear ERα present at PND 15 and TCDD delayed the presence of PR until PND 46. AR expression was very low in females and often variable, but DES-exposed animals very rarely had any expression at any time point. The male ERα, PR and AR staining data elucidates the differences and similarities between males and females during key moments of mammary gland development that previously have not been studied. Differences in steroid hormone receptor populations give insight into when EDCs would have their maximal effect on the mammary gland. ERα expression, while had very low overall expression levels in males, was present by PND 33, which is over a week later than it
is seen in females. PR expression was not detectable in the male mammary glands. AR expression was much higher in male mammary glands compared to females, and peaked at PND 46. DES treatment reduced the amount of AR expression at PND 46 compared to other treatments. The ranges of normal circulating serum hormone levels of nulliparous female rats starting at puberty and through adulthood are presented in Fig. 2.15.

The male rat mammary gland has not been extensively studied. The male mammary gland is structurally similar to the females up until puberty. While both glands grow exponentially, the male mammary gland grows at a slower rate than the females starting at PND 33 and stops growing in overall area after about PND 45, which coincides with the timing of male puberty and increased serum testosterone levels [36]. The female mammary epithelium continues to extend until it reaches the end of the fat pad, while the male mammary gland stops growing before then, most likely due to the animal reaching sexual maturity and increased levels of testosterone. Once the male reaches sexual maturity there is a structural change of the mammary gland from a tubuloalveolar to a lobuloalveolar structure, which has been previously described, but the precise mechanism for the shift in morphology is unknown [12, 37]. As expected the TCDD-exposed animals had developmentally delayed mammary gland growth before puberty, but seemed to catch up to controls by young adulthood. The DES-exposed animals did not exhibit accelerated mammary gland development compared to the controls. A different amount of DES or different DES exposure paradigm may be needed for an appreciable accelerated mammary gland development.

It is hoped that this atlas will enhance the ability of chemical screening and testing labs to produce and evaluate mammary gland sections or whole mounts from male and
female offspring in their studies. This atlas can be used as a reference for histological and gross changes that may occur in the situation of delayed or accelerated development. It may also provide useful information on mode of action for chemicals that shift steroid hormone receptor nuclear localization patterns. In order to prevent breast cancer in men and women and precocious breast development in girls around the globe, we must first understand some of the environmental factors adversely affecting the breast. Further evaluation of the male and female mammary gland in screening and test guideline studies will help produce the data needed to move this process forward.
Figure 2.1. Representative H&E images of female HSD rat fetal mammary gland development E15.5-17.5. (A and B) Mammary bud formation, E15.5 (A) 16X. (B) 40X. (C and D) Mammary stalk formation, E16.5 (C) 16X. (D) Mesenchymal layer forms (arrow) 40X. (E and F) Elongation of mammary stalk, E17.5. (E) 16X. (F) 40X.
Figure 2.2 Representative H&E images of female HSD rat fetal mammary gland development E18.5-21.5. (A and B) Invagination of the skin above the mammary gland, E18.5. (A) 16X. (B) 40X. (C and D) Elongation of the mammary gland, E19.5. (C) 16X. (D) 40X. (E and F) Formation of the nipple sheath, E20.5. (E) 16X. (F) Nipple sheath forms (arrow) 40X. (G and H) Primary duct begins lumen formation, E21.5 (G) 16X. (H) 40X.
Figure 2.3 Representative H&E images of male HSD rat fetal mammary gland development E15.5-17.5. (A and B) Mammary bud formation, E15.5 (A) 16X. (B) 40X. (C and D) Mammary stalk formation, E16.5 (C) 16X. (D) Mesenchymal layer forms (arrow) 40X. (E and F) Elongation of mammary stalk, E17.5. (E) 16X. (F) 40X.
Figure 2.4 Representative H&E images of male HSD rat fetal mammary gland development E18.5-21.5. (A and B) Atrophy of the mammary gland stalk, E18.5. (A) 16X. (B) 40X. (C and D) Further atrophy of the mammary gland stalk, E19.5. (C) 16X. (D) 40X. (E and F) Primary duct begins lumen formation, E20.5. (E) 16X. (F) 30X. (G and H) Elongation of the primary duct, E21.5. (G) 16X. (H) 40X.
Figure 2.5 H&E Images of female mammary gland development in control and gestationally exposed HSD rat offspring from birth to sexual maturity. (A-C) Mammary gland sections of neonatal rats on PND 4, 10X. (A) Control, (B) DES, and (C) TCDD-exposed. (D-F) Mammary gland sections of prepubertal rats on PND 21, 10X. (D) Control, (E) DES, and (F) TCDD-exposed. (G-I) Mammary gland sections of peripubertal rats on PND 46, 10X. (G) Control, (H) DES, and (I) TCDD-exposed. (J-L) Mammary gland sections of sexually mature rats on PND 70, 10X. (J) Control, (K) DES, and (L) TCDD-exposed.
Figure 2.6 H&E Images of male mammary gland development in control and gestationally exposed HSD rat offspring from birth to sexual maturity. (A-C) Mammary gland sections of neonatal rats on PND 4, 10X. (A) Control, (B) DES, and (C) TCDD-exposed. (D-F) Mammary gland sections of prepubertal rats on PND 21, 10X. (D) Control, (E) DES, and (F) TCDD-exposed. (G-I) Mammary gland sections of peripubertal rats on PND 46, 10X. (G) Control, (H) DES, and (I) TCDD-exposed. (J-M) Mammary gland sections of sexually mature rats change to a lobuloalveolar epithelial structure on PND 70, 10X. (J) Control, (K) DES lobuloalveolar structures (left) and remnant tubuloavleolar structures at the edge of the tissue (right), and (L) TCDD-exposed.
Figure 2.7 H&E-stained sections of HSD dam mammary gland during pregnancy and lactation. Control mammary glands during pregnancy and lactation (A) GD 15, (B) GD 17, (C) GD 21 and (D) PND 4. 20X.
Figure 2.8 ERα, PR and AR immunohistochemistry staining of control female mammary gland development. (A-C) ERα, (D-F) PR, and (G-I) AR. (A, D and G) PND 8, (B, E, and H) PND 33, and (C, F, and I) PND 70. 20X. There was nonspecific staining of the mast cells, not a part of the mammary gland epithelium.
Figure 2.9 ERα, PR and AR immunohistochemistry staining of control male mammary gland development. (A-C) ERα, (D-F) PR, and (G-I) AR. (A, D and G) PND 8, (B, E, and H) PND 33, and (C, F, and I) PND 70. 20X. There was nonspecific staining of the mast cells, not a part of the mammary gland epithelium.
Figure 2.10 ERα, PR and AR Quick Score of female and male offspring mammary glands. (A-B) (A) Nuclear ERα first appeared at PND 15 in the DES-exposed females and PND 21 in control and TCDD-exposed females. (B) Nuclear ERα first appeared at PND 33 in the males. (C) There was very little AR staining in the females and was sporadic in the chemically exposed animals and more consistent in the control females. (D) AR stained male glands at low levels until puberty in control and TCDD-exposed animals. DES had a less intense nuclear staining. (E) PR was only expressed in the females and first appeared at PND 33 in Control and DES-exposed animals, and PND 46 in TCDD-exposed animals. (n=2-3).
Figure 2.11 Whole mount female mammary gland developmental progression of control and prenatally exposed HSD offspring from birth to pre-puberty. Representative carmine stained mammary gland whole mount images of (A-C) neonatal rats on PND 1, 4X, (A) Control, (B) DES, and (C) TCDD-exposed; (D-F) neonatal rats on PND 8, 2X (D) Control, (E) DES, and (F) TCDD-exposed; (G-I) prepubertal rats on PND 21, 2X (G) Control, (H) DES, and (I) TCDD-exposed.
Figure 2.12 Whole mount female mammary gland developmental progression of control and prenatally exposed HSD offspring from puberty to sexual maturity. Representative carmine stained mammary gland whole mount images of (A-C) pubertal rats on PND 33, 0.8X, (A) Control, (B) DES, and (C) TCDD-exposed; (D-F) peri-pubertal rats on PND 46, 2X (D) Control, (E) DES, and (F) TCDD-exposed; and (G-I) adult rats on PND 70, 2X (G) Control, (H) DES, and (I) TCDD-exposed.
Figure 2.13 Whole mount male mammary gland developmental progression of control and prenatally exposed HSD offspring from birth to pre-puberty. Representative carmine stained mammary gland whole mount images of (A-C) neonatal rats on PND 1, 4X, (A) Control, (B) DES, and (C) TCDD-exposed; (D-F) neonatal rats on PND 8, 2X (D) Control, (E) DES, and (F) TCDD-exposed; and (G-I) prepubertal rats on PND 21, 2X (G) Control, (H) DES, and (I) TCDD-exposed.
Figure 2.14 Whole mount male mammary gland developmental progression of control and prenatally exposed HSD offspring from puberty to sexual maturity. Representative carmine stained mammary gland whole mount images of (A-C) prepubertal rats on PND 33, 0.8X, (A) Control, (B) DES, and (C) TCDD-exposed; (D-F) pubertal rats on PND 46, 2X (D) Control, (E) DES, and (F) TCDD-exposed; and (G-I) adult rats on PND 70, 2X (G) Control, (H) DES, and (I) TCDD-exposed.
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CHAPTER 3: VOC body burdens in rat mothers and offspring following drinking water exposures to a human relevant VOC mixture during pregnancy and adolescence

Introduction

Volatile organic compounds (VOCs) are common groundwater contaminates in many Superfund sites [1-5]. Superfund is the federal program to clean up uncontrolled hazardous waste sites in the U.S. and is committed to cleaning up hazardous waste sites to protect the environment and the health of local populations. VOCs will quickly off-gas from surface water but demonstrate a long half-life if they make their way into ground water. VOC contaminated groundwater used for human activities (bathing, cooking, watering plants, etc.) or consumption, can expose a population through inhalation, ingestion and dermal absorption. After exposure, VOCs are quickly eliminated from the body, as they have a short half-life in humans [1-5]. Populations near emission sources or contaminated drinking water could have elevated VOC serum levels in the low ppm range [1-6]. One example of accidental exposures to VOCs occurred on a Marine base in Jacksonville, NC, where VOCs contaminated some of the drinking water wells from the 1950’s to the 1980’s before the problem was identified and remediated. Hundreds of service people have complained of adverse health outcomes that they believed were linked to their VOC exposures [7]. Of the VOCs identified in the well water, benzene, vinyl chloride (VC) and trichloroethylene (TCE) are known human carcinogens, and tetrachloroethylene (PERC) is reasonably anticipated to be carcinogenic to humans [8-12].
In addition to the effects of the parent compounds, these VOCs are metabolized in the liver to form compounds known to be toxic [1-5]. The liver is an important organ as it functions to metabolize and detoxify many compounds. While some VOCs are eliminated by exhaling unconjugated forms (about 80% of PERC, 40% of benzene 10% of TCE and 5% of VC), extensive animals studies have revealed that VOCs are metabolized by phase one cytochrome P-450 (CYP) metabolism and phase two conjugation in the liver or kidneys. Benzene, PERC, TCE, trans-1,2-Dichloroethylene (trans-1,2-DCE) and VC are primarily metabolized by Cyp2e1. The many metabolic pathways for benzene are outlined by the Agency for Toxic Substances and Disease Registry (ATSDR) in their Toxicological Profile for Benzene. Cyp2e1 metabolism can result in the formation of the reactive metabolites 1,2- and 1,4-benzoquinone [1]. The concentrations of the individual metabolites of benzene are dependent on both the dose amount and route of exposure[13]. The toxicity of low-dose exposures to benzene may be underestimated by high-dose exposures.

TCE is metabolized by both CYP oxidation and glutathione (GSH) conjugation by GSH-tranferase [3]. Cyp2e1 is the major CYP responsible for the metabolism of TCE, but others (e.g., Cyp1a1/2, Cyp2b1/2 and Cyp2c11/6) can oxidize TCE when Cyp2e1 is not functional, as has been demonstrated using a knock-out Cyp2e1 mouse model [14]. TCE metabolite, trichloroacetic acid (TCA) modifies signaling pathways (peroxisome proliferation) resulting in cell death and reparative hyperplasia. Subsequent somatic mutations are thought to lead to liver toxicity and carcinogenesis [15]. The second pathway of TCE metabolism, GSH conjugation, produces S-dichlorovinyl-glutathione isomers that can be transported to the kidney and metabolized to S-dichlorovinylcysteine isomers. Both
types of isomers are mutagenic, transported to the kidneys, and play a significant role in the mutagenic mode of action in TCE-induced kidney tumors [8].

Both trans-1,2-DCE and cis-1,2-DCE are metabolized primarily by Cyp2e1 in the liver. Dichloroacetic acid (DCA) and 2,2-dichloroethanol are the major metabolites found after trans-1,2-DCE exposure and can be found in the urine [16]. The further metabolism of 2,2-dichloroethanol or DCA has not been investigated in the context of trans-1,2-DCE metabolism. However, it is known that the metabolites of trans-1,2-DCE do not undergo GSH conjugation[12, 16-17]. Exposure to trans-1,2-DCE does not elevate liver enzymes in rodents and it is not genotoxic [12]. The literature on rodents suggests that the liver is the organ most affected (fatty accumulation of liver lobules and Kupffer cells) by exposure to trans-1,2-DCE at high doses, but lacks evidence of any specific pathological event[12].

PERC is metabolized through two irreversible pathways: oxidation by Cyp2e1/Cyp2b1 and GSH conjugation via GSH-transferase [2, 10]. Oxidation of PERC can result in generation of the toxic metabolites TCA and dichloroacetic acid (DCA). As seen in TCE treated animals, liver cytotoxicity and carcinogenesis have been associated primarily with the metabolite TCA, while DCA may also play a role [18-19]. GSH conjugation of PERC primarily occurs in the liver and kidneys. Reactive metabolites in the kidneys produced via the glutathione conjugation pathway may play a role in the renal toxicity and carcinogenicity in PERC exposed rats [2, 10].

As stated in Chapter 1, previous testing of VOCs has primarily focused on high dose exposures to one chemical at a time in adult animals, a testing paradigm that may mask mammary gland effects. The goal of these studies were to better understand how VOCs behave under more human relevant conditions; namely as a mixture, in susceptible
subpopulations and through a drinking water exposure route. VOCs were mixed in sesame seed oil at ratios similar to well water at Camp Lejeune and then given as a single gavage dose to pregnant rats. Blood, mammary gland and a fetus were collected over 24 hours to access the half-lives of the VOCs in each of the three tissues. To determine how the VOCs would accumulate over time with an episodic VOC exposure in pregnant and adolescent rats, drinking water was given containing VOCs at concentrations 5-50 times higher than those reported at Camp Lejeune. The studies in Chapter 2 revealed the details of mammary gland development in male and female HSD rats. The studies in this chapter will determine the body burden complexities of VOCs when given as mixture, via drinking water exposure, in the pregnant/lactating HSD dam and her offspring.
Materials and Methods

Animals

Time pregnant Sprague Dawley rats were obtained from Harlan Laboratories (HSD; Indianapolis, IN) on gestational day (GD) 13 and allowed to acclimate for 2 days. Animals were weighed upon arrival and separated into groups with a similar average weight. Pregnant dams were housed individually in polypropylene cages with bedding and nestlets and switched to a rodent phytoestrogen-reduced diet (Zeigler; Madison, WI). Water and chow were supplied ad libitum. Animal facilities were maintained on a 12:12 h light-dark cycle at 20°C–23°C and 40–50% relative humidity. All animal studies were approved by the NIEHS Animal Care and Use Committee.

Chemicals

Chemical stock solutions for gavage dosing were purchased from Sigma Aldrich, (Steinhiem, Switzerland) and included; Benzene (0.874g/mL), PERC (1.622g/mL), TCE (1.463g/mL), DCE (1.252g/mL) and VC (2.0 g/mL). For the drinking water study, 1 ml ampules containing a concentrated standard mixture of benzene, PERC, TCE and trans-1,2-DCE were purchased by the Centers for Disease Control and Prevention (CDC) from O2si (Charleston, SC) and VC (2000µg/ml) was purchased from Sigma Aldrich (Steinhiem, Switzerland). The measured level of VOCs in the well water at Camp Lejeune [20] was denoted as 1 X: 1.5ppm PERC, 0.2ppm TCE, 0.4ppm trans-1,2-DCE, 0.025ppm VC and 0.2ppm benzene. VOC drinking water solutions for this study were prepared by spiking 38.85 µl of the mixture standard and 312.5 µl of VC into 500 ml deionized/reverse osmosis water resulting in 10 µg/ml of benzene, 75 µg/ml of PERC, 10 µg/ml of TCE, 20 µg/ml of trans-1,2-DCE and 1.25 µg/ml of VC for the “50X” or 50 times the well water VOC.
concentrations. Serial dilutions were made for “10X” and “5X” concentrations (Table 3.1). 500 ml glass drinking water bottles with metal sippers were filled with dosed drinking water and given to animals to drink ad libitum. Pilot experiments showed that VOC off-gassing depended on the amount of head space in the water bottle more so than the length of time since freshly mixed (data not shown). The drinking water bottles were changed at least every other day to ensure no more than 25% of the water was consumed before changing the water, to reduce variation in VOC water concentration.

Experimental Design

Two animal studies were conducted with the mixture of VOCs.

1. **Half-life of a mixture of VOCs in the pregnant dam:** Time pregnant HSD rats (18 dams) arrived at the NIEHS on GD 13 (GD 0=plug positive). On GD 15 each animal (except one non-treated control) was gavage dosed with a mixture of VOCs at 0.25mg/kg VC, 20mg/kg benzene, 20mg/kg PERC, 40mg/kg DCE and 150mg/kg TCE at a volume of 5 mL/kg body weight in sesame oil (Table 3.1). Rats were weighed just prior to dosing. High doses were selected to insure half-lives could be estimated and the ratio of chemicals given were based on well water at Camp Lejeune.

2. **Body burden from VOCs in drinking water:** Timed-pregnant HSD rats (68 dams) arrived on GD 10, were switched to a low soy Purina Lab Diet 5008 chow and were divided into 4 treatment groups (n=13, 18, 17 and 19 in Control, 5X, 10X and 50X VOC dose groups, respectively). All dams and pups were given glass water bottles containing concentrations of the VOC mixture (0, 5X, 10X or 50X VOC concentrations, see Table 3.1) from GD 12 through PND 48 from
which they could drink ad libitum. Pups began drinking water exposure on PND 13 (once their eyes opened). Dams were allowed to give birth and litters were equalized on PND 4 to 5 female and 5 male offspring. Drinking water exposure continued for dams and pups until sacrifice.

Necropsy

1. **Half-life of a mixture of VOCs in the pregnant dam**: Two dams per time point were sacrificed by CO₂ affixation at 0, 15mins, 30mins, 2 hrs, 4hrs, 8hrs, 16hrs and 24hrs after gavage with VOC mixture. Dams were sacrificed for VOC blood concentration. To evaluate the amount of VOC present in the blood it was important to prevent any off-gassing of VOC when collecting the blood and to prevent potential VOC contamination. Tubes, stoppers and syringes were provided by the CDC to insure there were no trace VOC residues. The rats were sedated and a terminal cardiac puncture was performed. The blood was collected directly into blood collection tubes so that it was not exposed to the room air. A section of the 4ᵗʰ mammary gland and one intact fetus were also collected and placed in separate blood collection tubes. After collection, samples were refrigerated and then shipped cold with freezer packs, but not frozen, by overnight delivery to CDC laboratories in Atlanta, GA for analysis.

2. **Body burden from VOCs in drinking water**: Three dams from each dose group were sacrificed on GD 15, GD 17, GD 20, PND 15 and PND 21 by CO₂ affixation. Three female and three male pups from each dose group were sacrificed on PND 15, 21, 28 and 48 by CO₂ affixation. Blood was collected and shipped to the CDC in a similar fashion as the half-life study. For an initial
analysis, the blood of dams (GD13-21) in the control, 5X and 10X dose groups were tested. It was determined that the 5X group was too close to the lower limit of detection, so for the rest of the blood (dams PND 15, 21 and pups PND 15, 21, 28, 48) only 10X and 50X were tested. Body weights and liver weights were recorded. For necropsies on the offspring, after the blood was obtained, one set of 4th and 5th mammary glands were removed for whole mount analysis and the contralateral set was collected, placed in histology cassettes and fixed in formalin for IHC processing. A section of liver and mammary gland were snap frozen for RNA analysis.

*Headspace solid-phase microextraction with benchtop gas chromatography–mass spectrometry*

To quantify levels of VOCs, blood samples were measured by headspace solid-phase microextraction (SPME)/gas chromatography (GC)/isotope dilution mass spectrometry (MS) using similar methods as described by Blount, et al.[21] and Chambers, et al.[22]. Analysis of the blood sample was performed by equilibrium headspace analysis using SPME. For analysis, 3-ml of blood was transferred by gas-tight syringe from a blood collection tube to a headspace vial. The SPME fiber shaft was inserted into the headspace of a hermetically sealed sample vial containing the blood sample. The VOCs partitioned into the coating on the outside of the SPME fiber shaft. This fiber was then inserted into the heated GC inlet where the VOCs rapidly desorbed because of the high temperature. Extracted VOCs were focused at the head of the GC column using a cryogenic trap. Analytes were separated on a capillary column designed for VOC analyses and quantified using MS (unit mass resolution). Response calibration was performed using standards to normalize calibration standards and
blood sample response. This method is applicable to the determination of a broad range of VOCs with detection limits in the low-parts-per-trillion range. Similar methods were used to analyze fetal and mammary gland tissues. The detection limits achieved with this method were 5, 24, 5, and 8 pg/ml for trans-1,2-DCE, benzene, TCE, and PERC, respectively.

Quantitative Polymerase Chain Reaction

Frozen liver samples from male offspring were homogenized by MP FastPrep-24 homogenizer using lysing matrix D (MP Biomedicals, Solon, OH) in TRIzol (Life Technologies Inc, Rockville, MD) and RNA was isolated according to TRIzol manufacturer’s protocol. Total RNA was cleaned-up using Qiagen RNeasy Mini Kit with the addition of On-Column DNase digestion (Qiagen; Hilden, Germany) according to manufacturer’s instructions. Total RNA was quantified using NanoDrop 2000c (ThermoScientific, Wilmington, DE). Taqman (Applied Biosystems, Branchburg, NJ) assays were purchased for Cyp2e1, Cyp2b1 and Actb. Approximately 2μg RNA was reverse transcribed using the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). 50 ng of cDNA was run in duplicate on QuantStudio 7 Flex Real Time Instrument (Applied Biosystems). Cycle Threshold (Ct) values were calculated using QuantStudio Real Time Software and were included in the analysis if the standard error for the duplicates were ≤0.5. The ΔCt was calculated for each transcript by normalizing the sample Ct values by the Ct values of Actb transcript in the same sample. For VOC treatment effect, the relative gene expression was calculated by dividing the mean 2-Δct of treated samples over the mean 2-Δct of the control. For CYP changes over time, the relative gene expression was calculated by dividing the mean 2-Δct of PND 21, 28 and 48 samples over the mean 2-Δct of the PND 14
samples. For relative gene expression that was between zero and one, the negative inverse was used to represent fold change.

**Estimation of rates of elimination and half-life calculations of VOCs**

Rates of elimination of VOCs were estimated as described in Troester et al. (2000) [23]. Following a single dose of VOC mixture, individual chemicals were estimated to be eliminated by first-order kinetics according to the following expression:

$$[A]_t = [A]_0 e^{-\lambda t}$$

Where $A_0$ is the chemical concentration at $t=0$ and $\lambda$ (d$^{-1}$) is the first-order rate constant for each chemical’s elimination. From the above equation, the relationship between $\ln[A(t)]$ and $t$ is linear with intercept = $\ln(A_0)$ and slope = $-\lambda$, that is,

$$\ln[A]_t = \ln[A]_0 - \lambda t$$

And $\lambda$ can be estimated by linear regression of $\ln A(t)$ on $t$. Half-lives ($t_{1/2}$) of each chemical were then determined by the following equation:

$$t_{1/2} = \frac{0.693}{\lambda}$$

To determine $\lambda$, first peak concentration was determined. Then time of collection ($t$) was transformed into time from peak concentration ($t'$). The concentrations (conc) were transformed into the $\ln[\text{conc}]$. In Excel, the coordinates of $t'$ and the $\ln[\text{conc}]$ of each time point from peak concentration were plotted on a graph and a line was fitted to the resulting coordinates. The slope of the fitted line was $-\lambda$ and was entered into the above question for $t_{1/2}$. 
\textit{Percent daily dose per gram tissue estimations for 10X dams}

Direct comparisons between the amounts of each VOC in the blood was not possible, since the concentrations of each VOC in the drinking water were different. Measuring the amount of water consumed during the study was also not possible because once born the animals were grouped house for the duration of the study. Since direct calculations of average daily doses were not possible, an estimated average daily dose was used instead. The assumption was made that for each day of pregnancy, average water consumption would result in an average daily dose of $\frac{1}{8}$th the VOC concentration in the water, and during lactation it was $\frac{1}{5}$th. The estimated average dose (mg/kg) was determined by dividing the concentrations of VOC in the drinking water (mg/L) by the conversion estimate for the specific life stage (8 during pregnancy or 5 during lactation). For example, an 80mg/L water concentration would result in a 10mg/kg average daily dose for the dam each day of pregnancy and 16mg/kg average daily dose each day of lactation. These estimates may over or under shoot actual average daily doses as water consumption changes over time, but these calculations allow for a percent dose per gram tissue to be calculated. The conversion estimates were based on studies performed at the NTP on HSD pregnant and lactating rats (personal communication Michael DeVito, 11/24/2014). Average daily doses could only be estimated for the dams because conversion estimates exist for the dam during both pregnancy and lactation and do not exist for pups due to their rapid and variable growth rate depending on litter size and sex ratios of the litter. The estimated average daily dose was then multiplied by the weight of the dam, which was measured in this study, resulting in the amount of VOC ingested daily (mg). Then the percent daily dose per ml of blood was estimated by dividing the amount of VOCs measured in the blood divided by the estimated daily dose. For percent
dose, the amount measured in the blood was divided by the cumulative amounts of the VOCs ingested over time. Transforming the amount of each VOC in the blood to a percent of daily dose allows for the estimated comparison of dose between the VOCs in a mixture.

**Statistical Analysis**

For qPCR analysis, data was analyzed in Microsoft Excel and statistical significance was determined with Student’s t-tests. Significance was reported for p-values ≤ 0.05.
Results

Half-life determination

There were three goals of the first study: 1) To determine if VOC levels in blood collected under hermetic conditions and tissues of interest could be measured, 2) to define any potential changes in half-life of the VOCs when given as a mixture to a pregnant rat, and 3) determine if VOCs were being transferred to the developing fetus following oral intake from the dam. To accomplish these goals, whole blood, mammary gland and a fetus was analyzed for changes in VOC concentration over a 24 hour period. Table 3.2 reports the half-lives of each chemical in blood, mammary gland and fetus. VC is not included since the only established protocol for VC detection at the CDC is in urine. Urine was not collected because the methods of euthanasia of the dams resulted in complete emptying of the bladder before it could be collected under hermetic conditions. Half-lives of the four VOCs in the blood of timed-pregnant rats mirrored those previously reported [1-4] following single chemical exposure in adult non-pregnant rats; the chemicals are eliminated quickly after gavage dose. The VOC with the longest half-life in the blood of a pregnant dam was PERC (about 5 ½ hr), followed by approximately 2 hr half-lives for the other chemicals. The data suggests that the half-lives of these chemicals are longer in the mammary gland of the dam and the fetus compared to the blood of the dam. Most of the K_{ow} for these chemicals suggest they are slightly lipophilic (Tables 1.1 through 1.5) and are expected to reside longer in the mammary fat pad, potentially exposing mammary gland epithelium during critical periods of development in both the dam and the fetus and may be transferred to the milk. Importantly, VOCs can cross the placenta and are detected in the fetus, where it is estimated to have a longer half-life than in the blood of the dam.
**Drinking water body burden**

The VOCs were shown to have a short half-life in the gavage study and the next question was, “Would lower dose drinking water exposure result in a constant level of chemical exposure or would the dosing be more of an episodic exposure?” Dams and their resulting offspring were given drinking water that was either untreated or treated at 5X, 10X or 50X VOCs from GD 12-PND 48. Dam VOC exposure would have primarily come from drinking water exposure, but also potentially from ingesting urine and feces when grooming the young pups. Pups were exposed to VOC though gestational and lactation exposure and also by drinking water exposure when their eyes were open and they were big enough to reach the water bottle. A mixture of lactational and drinking water exposure occurred until they were weaned from the dams on PND 21 and then their only exposure was from drinking treated water.

The body burden data of the dam and pup is shown in Fig. 3.1 and 3.2, respectively. The figures suggest an episodic exposure to the VOCs; there is no accumulation over time with repeated doses per day. Spikes in blood concentration coincided with increased levels of water consumption due to lifestage (i.e., peak lactation highest; day of partition lowest). Large variability within some time points suggest one animal drank more recently that the others. PERC was given at the highest concentration and had the longest half-life of the chemicals tested. Therefore, it was present at the highest concentration at most time points. TCE and DCE remained at levels similar to background found in untreated dams. Surprisingly, benzene concentrations were higher than background and in the case of 10X pups were at higher or around the same concentration as PERC. The concentrations of
benzene and TCE in the drinking water were the same, while the concentration of trans-1,2-DCE was two times higher than benzene and TCE. There was also an initial higher than expected amount of benzene in the blood at PND 15 for both the 10X and 50X dosed animals that decreased with age. A shift in VOC metabolism over time or a novel mechanism for benzene metabolism may be responsible for the high levels of benzene in the blood compared to the rest of the VOCs.

*Changes in percent dose per gram body weight and percent daily dose per gram body weight.*

Percent dose and percent daily dose were calculated so that comparisons of VOC found in the blood could be made. By comparing the percent dose found in the blood, it removes the differences in water concentrations of each chemical. The percent of PERC in the blood can be compared to the percent of TCE, without having to consider the higher concentration of PERC in the drinking water. Percent dose levels in the Table 3.3 shows the percent dose of each chemical in the 10X dam when the VOC were consumed as a mixture in water. Percent dose was calculated based on the percent of the VOCs detected in 1 ml of blood, compared to the sum of all estimated daily doses. The percent dose in blood was less than 1% for all chemicals after one day of exposure. PERC and benzene had similar percent dose over time. TCE and trans-1,2-DCE had low percent dose values through the entire study. Because of the similar half-lives, benzene was expected to be comparable to DCE and TCE. PERC has a longer half-life and would be expected to have a higher percent dose than the other chemicals. With time, the percent dose typically decreased and by lactation had decreased to less than 0.001% for all chemicals, suggesting that these levels of VOC do not accumulate in the blood over time.
The percent daily doses in the 10X dam are listed in Table 3.4. Percent daily dose does not take in to account the total accumulation of VOCs from the start of exposure, but only the percent of the average daily dose of that timepoint found in the blood. After one day, PERC had the highest percent daily dose (0.066%), followed by benzene with a slightly lower percent daily dose (0.05%). TCE and trans-1,2-DCE percent daily doses were barely detectable (0.001%). After three days of exposure, the percent daily dose increased for all VOCs and then dropped back down at GD 21 (parturition), likely due to the dramatic changes in water intake. During lactation, the percent daily dose increased to the highest levels for all VOCs on PND 15, the peak of lactation, and then were back down to levels similar to the first day of dosing by weaning. Again, these changes were in line with expected water intake levels.

**CYP mRNA expression in liver from water exposure studies.**

Decreasing benzene concentrations over time were noted in the blood of offspring exposed to 10X and 50X concentrations of VOC. A possible explanation could be that VOCs are increasing the expression of CYP enzymes with increasing age and metabolizing the benzene at a faster rate. To determine whether VOC exposure induced changes in CYP expression pathways responsible for VOC metabolism (Cyp2e1 and Cyp2b1), livers were evaluated from male pups on PND 15, 21, 28 and 48. Fold change gene expression for Cyp2e1 and Cyp2b1 are shown in Table 3.5 and 3.6. There were few dose and/or dose/time-dependent gene changes found in the tissues evaluated. VOC exposure significantly down-regulated expression of Cyp2e1 at PND 48, at the 10X concentration. The same effect was seen in Cyp2b1 expression; there was a significantly down-regulated expression of Cyp2b1 at PND 48 at the 10X concentration. When looking at CYP expression over time, in Tables
3.7 and 3.8, there was a significant down-regulation in Cyp2e1 at PND 48 in control, 10X and 50X dose group compared to PND 15. There was a significant down-regulation in Cyp2b1 expression at PND 48, when compared to PND 15, at the 10X concentration. This shift in expression over time suggests that Cyp2e1 has higher expression during neonatal/prepubertal time points. Similar effects in Cyp2e1 activity changes have been observed other strains of rat [24].
Discussion

A gavage dose of the VOC mixture allowed for a validation in the collection and detection protocols, a half-live determination in tissues of interest, and confirmation that VOCs were transferred to mammary tissue (and potentially milk) and the developing fetus. These were important premises to establish for the rest of the studies I propose. The blood half-lives of the VOCs were comparable to the half-lives previous determined in non-pregnant rats [1-5]. Benzene, at high concentrations has an initial rapid excretion phase (41 mins) followed by a slower elimination rate. At low exposure concentrations (6.4 ppm), a less rapid elimination rate is seen (1.2 hours) [1] and this closely matches the findings in the pregnant dam in these studies of a half-life of 1.6 hours. In rats, PERC has been shown to have a half-live of 5.3 hours in the blood and 9.6 hours in the fat [2]. The half-life of PERC in the blood of pregnant rats (5.7 hr) matched closely to previous findings in non-pregnant rats and in the mammary gland, PERC had a longer half-life (6.8 hr), closer to the previous reported half-life in fat of 9.6 hours. The half-life of PERC reported in humans can be much longer than in the rat, especially in the fat; up to 55 hours [2]. TCE has been reported to have a short half-life in non-fasted rats of around 3 hours verses 2 hours in fasted rats [3]. The pregnant dams in this study were not fasted but had a half-life similar to previously studied fasted male rats (2 hr) [25]. trans-1,2-DCE has not been as extensively studied as the other three chemicals, cis-1,2-DCE has an estimated half-life of less than one hour in the blood of humans [12]. The present study demonstrated that in the pregnant rat the half-life of DCE in the blood is just over an hour and a half and is closer to two hours in the mammary gland. VC could not be detected in the blood and urine analysis would be necessary to make half-life estimations. Because of the nature of the euthanasia of the animals (CO₂ knockdown and
then terminal cardiac puncture) the bladders were emptied by the time of necropsy and urine could not be collected without exposure to the air and potential loss of VOCs due to volatilization.

VOCs were detected in both the mammary gland and fetus, thus it is reasonable to assume that the offspring were exposed to VOC both transplacentally and from ingesting contaminated milk from the dam, in addition to ingestion of VOC-laced drinking water once they could reach the water. The half-lives of VOCs in the fetus compared to the blood or mammary gland varied with each chemical. The fetal half-lives were slightly longer than in the dam with the exception of PERC, where the high-life was approximately the same. The half-lives of the chemicals found in the blood of the pregnant rat did not vary from what have previously seen in the adult non-pregnant rat and as anticipated, these chemicals have a longer half-life in fatty tissue; the mammary gland. These VOC are mildly lipophilic, as evidenced by $K_{ow}$ in Chapter 1, but the mammary gland does not have a very high perfusion rate compared to other tissues [26]. PERC can accumulate in humans [2], and it was important to see if blood levels of VOC would ever reach a plateau or steady state.

Considering the episodic nature of the exposure from drinking water, the low concentrations given, and the short chemical half-lives, VOC concentrations in the rat were not expected to and did not reach a steady state exposure. The concentrations found in the blood were likely dependent on the amount of water consumed and how recently before necropsy they last drank, neither of which was exactly known.

Blood concentrations of PERC were expected to be the highest among the chemicals measured in all treated groups, as PERC was at the highest concentration in the VOC mixture (Table 3.1). Blood concentrations of PERC in the dams were the highest concentration of any
of the other compounds. Blood concentrations of benzene, TCE and trans-1,2-DCE were close to background levels for the majority of animals (with the exception of 50X treated dams and 10X treated dams at PND 21). Blood concentrations of benzene were the second highest in all the treated groups and were up to 10 times higher than TCE or trans-1,2-DCE. This was surprising since the half-lives and the concentrations of benzene and TCE in the drinking water are approximately equal. Even more so that the half-lives of benzene and trans-1,2-DCE are also equal yet there is twice the concentration of trans-1,2-DCE in the drinking water than benzene. Benzene had an even more elevated presence in the pups. In the 50X dosed offspring, PERC was in general much higher than the other chemicals, with the exception of benzene. At PND 15, benzene levels were elevated to concentrations similar to PERC. PERC blood levels generally increased with age, while benzene levels decreased with age. In the 10X exposed offspring, blood benzene levels were higher than PERC until PND 48, despite being found at 1/15th the amount in the water. Like the 50X treated animals, benzene blood levels decreased with age. TCE and trans-1,2-DCE also remained at background levels in the offspring, with only one slight peak in trans-1,2-DCE levels in the 50X offspring at PND 21. The benzene trends were unexpected.

Since the concentrations of each VOC in the drinking water varied, an estimated percent dose and percent daily dose calculations in the dam were made to be able to compare the VOC exposures. Actual percent dose and percent daily doses cannot be determined because the drinking water consumption of each rat is unknown. Water consumption was not recorded because, once the pups could start drinking water and after weaning when the offspring were group housed, it would be unknown how much water each individual consumed. The NTP has performed many studies testing chemical exposure both through
drinking water and diet. Exact water and food consumption in studies where animals are group housed (reproductive assessment by continuous breeding, 2-Year bioassay and 13-week exposure studies) cannot be determined and so conversion estimates were developed to roughly estimate average daily dose in the HSD rats. The conversion estimates where determined by comparing the concentration in the water or diet and amounts of chemical actually in the animal. The conversion estimates allow for an estimation of average daily dose from drinking water or diet exposures when designing a dose-range finding study. The conversation estimates make some assumptions, namely that the conversion estimate is 8 between GD 9-21 and 5 during lactation and that the concentration of a chemical in the water or diet will not change over time. By changing the water frequently, variability in VOC concentrations was minimized. Despite being potentially inaccurate, the estimated percent dose and daily dose allowed for the comparisons between the VOCs and revealed both anticipated and unanticipated results. Comparing the VOC half-lives to their percent doses and percent daily dose, benzene, TCE and trans-1,2-DCE were expected to have similar percent doses and percent daily doses, but benzene was 10-50 times higher during pregnancy. Some property of benzene or the VOCs as a mixture makes the percent dose and percent daily dose of benzene in the blood higher than TCE or trans-1,2-DCE, despite similar half-lives, amount in the dosing solution and $K_{ow}$.

The major enzyme for metabolizing benzene, PERC, TCE, trans-1,2-DCE and VC in the liver is Cyp2e1 [1-5]. Elimination of benzene may be affected by the mixture of VOCs that makes the percent dose so much higher than TCE or trans-1,2-DCE. PERC has the longest half-life of all the chemicals and was expected to have the highest percent dose at all time points.
Major metabolism of these chemicals in the liver are dependent on Cyp2e1 (Benzene, PERC, TCE, trans-1,2-DCE and VC) and Cyp2b1 (PERC). The ontogeny of CYP liver enzyme expression (measured either by mRNA expression, protein levels and/or activity) in rats from gestation through adulthood has previously been summarized [24]. For both Cyp2e1 and Cyp2b1 there is very low hepatic expression and/or activity during gestation. Fetuses depend on maternal metabolizing enzymes until birth. Cyp2b1 expression in the liver of the pups is higher than adult levels from parturition until weaning. Cyp2e1 expression remains low the first few days after birth and becomes higher than adult levels until weaning [24]. In this study, the control offspring were expected to start with high expression at PND 15 and then CYP levels would decrease by later time points to the normal adult expression levels. Because of the limited amount of liver samples collected only mRNA was collected and analyzed by qPCR for CYP expression. When comparing CYP expression over time, we detected the expected significant down regulation of Cyp2e1 in the Control, 10X and 50X dosed animals at PND 48, compared to PND 15. The same significant down regulation of Cyp2b1 expression was found in the 10X dosed animals, but there was no difference in the control, 5X or 50X dosed animals over time. VOC exposure did not significantly change CYP expression for the majority of treated animals, and changes in CYP expression cannot explain the unexpectedly high levels of benzene in pup blood.

Although activity was not directly measured, it seems unlikely that low levels of VOCs given in this study would alter the activity of CYPs to a significant level. There is a possibility that the blood benzene levels drop due to dilution in the quickly growing pup, but a similar drop in PERC would have also been expected. Some of the limitations of these studies come from the desire to closely recapitulate water exposure at Camp Lejeune. Future
studies would benefit from recording water consumption for the dams before the pups can reach the water bottles. Future studies should also examine the effects of the mixture of VOCs on the activity of the CYP enzymes. Rats exposed to 40 ppm trans-1,2-DCE before VC or TCE exposure were reported to have reduced metabolism due to trans-1,2-DCE competitive inhibition of Cyp2e1 [27]. The low levels of the VOCs in mixture do not induce a change in mRNA expression but may compete for Cyp2e1, altering metabolism of the mixture.

In the previous chapter, it was important to determine how the mammary gland development of the HSD rat was affected under normal and chemically altered conditions. In this chapter, these studies determined how each VOC behaves: 1) in a gestational/lactational drinking water exposure, where body weights change daily and 2) as a mixture with other similarly metabolized and excreted VOCs. The toxicokinetics of these individual chemicals have mostly been studies in adult animals, where body weights and compartment sizes don’t fluctuate significantly. These studies are focused on human relevant exposures to VOCs when consuming contaminated drinking water. It was important to understand and recapitulate the VOC exposure in pregnant mothers and children/young adult at sites like Camp Lejeune. With a general understanding of the mammary gland development and VOC body burden in HSD rat, Chapter 4 will assess VOC exposure on the mammary gland.
Table 3.1 VOC concentrations in well water at Camp Lejeune, in drinking water studies, and in the 100X gavage dose used in the half-life study

<table>
<thead>
<tr>
<th></th>
<th>PERC</th>
<th>TCE</th>
<th>trans-1,2 DCE</th>
<th>VC</th>
<th>Benzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>ppm in well water [20]</td>
<td>1.5</td>
<td>0.2</td>
<td>0.4</td>
<td>0.025</td>
</tr>
<tr>
<td>5X</td>
<td>μg/ml in water</td>
<td>7.5</td>
<td>1</td>
<td>2</td>
<td>0.125</td>
</tr>
<tr>
<td>10X</td>
<td>μg/ml in water</td>
<td>15</td>
<td>2</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>50X</td>
<td>μg/ml in water</td>
<td>75</td>
<td>10</td>
<td>20</td>
<td>1.25</td>
</tr>
</tbody>
</table>

**Half-life study** | mg/kg in sesame oil | 150  | 20            | 40    | 2.5     | 20      |

Note: VOC= volatile organic compound. HSD= Harlan Sprague Dawley. GD= gestational day. PERC= tetrachloroethylene. TCE= trichloroethylene. trans-1,2-DCE= trans-1,2-dichloroethylene. ppm is equivalent to μg/ml.
Table 3.2 Calculated VOC half-lives (in hours) in pregnant HSD rat and fetus at GD 15

<table>
<thead>
<tr>
<th>Dam</th>
<th>Benzene</th>
<th>PERC</th>
<th>TCE</th>
<th>trans-1,2 DCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1.61</td>
<td>5.62</td>
<td>1.99</td>
<td>1.52</td>
</tr>
<tr>
<td>Mammary Gland</td>
<td>2.12</td>
<td>6.77</td>
<td>2.22</td>
<td>1.98</td>
</tr>
<tr>
<td>Fetus</td>
<td>2.34</td>
<td>5.61</td>
<td>2.12</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Note: VOC= volatile organic compound. HSD= Harlan Sprague Dawley. GD= gestational day. PERC= tetrachloroethylene. TCE= trichloroethylene. trans-1,2-DCE= trans-1,2-dichloroethylene.
Table 3.3 Percent dose per 1 ml blood in the 10X HSD dam

<table>
<thead>
<tr>
<th></th>
<th>Benzene</th>
<th>PERC</th>
<th>TCE</th>
<th>trans-1,2 DCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD 13</td>
<td>0.050%</td>
<td>0.066%</td>
<td>0.001%</td>
<td>0.001%</td>
</tr>
<tr>
<td>GD 15</td>
<td>0.016%</td>
<td>0.022%</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
</tr>
<tr>
<td>GD 21</td>
<td>0.007%</td>
<td>0.026%</td>
<td>0.001%</td>
<td>&lt;0.001%</td>
</tr>
<tr>
<td>PND 15</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
</tr>
<tr>
<td>PND 21</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
<td>&lt;0.001%</td>
</tr>
</tbody>
</table>

Note: HSD= Harlan Sprague Dawley. PERC= tetrachloroethylene. TCE= trichloroethylene. trans-1,2-DCE= trans-1,2-dichloroethylene. GD= gestational day. PND= postnatal day. Calculated as: the amount of VOC measured in 1 ml of blood divided by the sum of the estimated average daily mg dose with exposures starting on GD 12. The average daily dose was estimated by dividing the concentration of VOCs in the drinking water by a conversion estimate (8 for GD 12-21 and 5 for PND 1-21) and multiplying by the average body weight of the dam on the day of blood collection.
<table>
<thead>
<tr>
<th></th>
<th>Benzene</th>
<th>PERC</th>
<th>TCE</th>
<th>trans-1,2 DCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD 13</td>
<td>0.050%</td>
<td>0.066%</td>
<td>0.001%</td>
<td>0.001%</td>
</tr>
<tr>
<td>GD 15</td>
<td>0.057%</td>
<td>0.226%</td>
<td>0.005%</td>
<td>0.004%</td>
</tr>
<tr>
<td>GD 21</td>
<td>0.041%</td>
<td>0.090%</td>
<td>0.002%</td>
<td>0.002%</td>
</tr>
<tr>
<td>PND 15</td>
<td>0.105%</td>
<td>0.179%</td>
<td>0.106%</td>
<td>0.043%</td>
</tr>
<tr>
<td>PND 21</td>
<td>0.027%</td>
<td>0.110%</td>
<td>0.001%</td>
<td>0.004%</td>
</tr>
</tbody>
</table>

Note: HSD = Harlan Sprague Dawley. PERC = tetrachloroethylene. TCE = trichloroethylene. trans-1,2-DCE = trans-1,2-dichloroethylene. GD = gestational day. PND = postnatal day. Calculated as: the amount of VOC measured in 1 ml of blood divided by the estimated average daily mg dose. The average daily dose was estimated by dividing the concentration of VOCs in the drinking water by a conversion estimate (8 for GD 12-21 and 5 for PND 1-21) and multiplying by the average body weight of the dam on the day of blood collection.
Table 3.5 Fold change of Cyp2e1 mRNA expression in VOC treated offspring

<table>
<thead>
<tr>
<th></th>
<th>PND 15</th>
<th>PND 21</th>
<th>PND 28</th>
<th>PND 48</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X</td>
<td>-1.62</td>
<td>-1.43</td>
<td>1.01</td>
<td>1.80*</td>
</tr>
<tr>
<td>10X</td>
<td>1.26</td>
<td>-1.14</td>
<td>1.04</td>
<td>-1.01</td>
</tr>
<tr>
<td>50X</td>
<td>1.25</td>
<td>-1.55</td>
<td>-1.37</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Note: VOC= volatile organic compound. PERC= tetrachloroethylene. TCE= trichloroethylene. trans-1,2-DCE= trans-1,2-dichloroethylene. PND= postnatal day. Significant effects compared to controls by Student’s t-test, *p<0.05. mRNA expression in control rat is 1.
Table 3.6 Fold change of Cyp2b1 mRNA expression in VOC treated offspring

<table>
<thead>
<tr>
<th></th>
<th>PND 15</th>
<th>PND 21</th>
<th>PND 28</th>
<th>PND 48</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X</td>
<td>-1.13</td>
<td>1.14</td>
<td>-1.49</td>
<td>-1.68</td>
</tr>
<tr>
<td>10X</td>
<td>1.25</td>
<td>1.12</td>
<td>-1.44</td>
<td>-2.20*</td>
</tr>
<tr>
<td>50X</td>
<td>-1.13</td>
<td>-1.16</td>
<td>1.07</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Note: VOC= volatile organic compound. PERC= tetrachloroethylene. TCE= trichloroethylene. trans-1,2-DCE= trans-1,2-dichloroethylene. PND= postnatal day. Significant effects compared to controls by Student’s t-test,*p≤0.05. mRNA expression in control rat is 1.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5X</th>
<th>10X</th>
<th>50X</th>
</tr>
</thead>
<tbody>
<tr>
<td>PND 21</td>
<td>1.37</td>
<td>1.55</td>
<td>-1.05</td>
<td>-1.04</td>
</tr>
<tr>
<td>PND 28</td>
<td>1.25</td>
<td>2.04</td>
<td>1.03</td>
<td>-1.37</td>
</tr>
<tr>
<td>PND 48</td>
<td>-2.05*</td>
<td>1.43</td>
<td>-2.61*</td>
<td>-2.00*</td>
</tr>
</tbody>
</table>

Note: VOC = volatile organic compound. PERC = tetrachloroethylene. TCE = trichloroethylene. trans-1,2-DCE = trans-1,2-dichloroethylene. PND = postnatal day. Significant effects compared to PND 15 by Student’s t-test, *p ≤ 0.05. mRNA expression in PND 15 rat is 1.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5X</th>
<th>10X</th>
<th>50X</th>
</tr>
</thead>
<tbody>
<tr>
<td>PND 21</td>
<td>-1.35</td>
<td>-1.05</td>
<td>-1.50</td>
<td>1.46</td>
</tr>
<tr>
<td>PND 28</td>
<td>-1.10</td>
<td>-1.45</td>
<td>-1.96</td>
<td>1.72</td>
</tr>
<tr>
<td>PND 48</td>
<td>1.07</td>
<td>1.70</td>
<td>-2.55*</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Note: VOC= volatile organic compound. PERC= tetrachloroethylene. TCE= trichloroethylene. trans-1,2-DCE= trans-1,2-dichloroethylene. PND=postnatal day. Significant effects compared to PND 15 by Student’s t-test,*p≤0.05. mRNA expression in PND 15 rat is 1.
Figure 3.1 VOC blood concentrations (ng/ml) in dams exposed to VOC mixture in drinking water. (A) Control dam. (B) 5X dam. (C) 10X dam. (D) 10X dam with PERC removed. (E) 50X dam. (F) 50X dam with PERC removed. The detection limits achieved with this method were 5, 24, 5, and 8 pg/ml for 1,2-DCE, benzene, TCE, and PERC, respectively.
Figure 3.2 VOC blood concentrations (ng/ml) in offspring exposed to VOC mixture in drinking water. (A) 10X offspring. (B) 50X offspring. The detection limits achieved with this method were 5, 24, 5, and 8 pg/ml for trans-1,2-DCE, benzene, TCE, and PERC, respectively.
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CHAPTER 4: Mammary developmental effects and tumor susceptibility in Harlan Sprague Dawley rats following in utero and adolescent exposures to a VOC mixture in drinking water

INTRODUCTION

Volatile organic compounds (VOCs) are a subset of organic compounds with physical and chemical properties that allow them to move between water and air. VOCs tend to have high vapor pressures, low-to-medium water solubility, and low molecular weights. Some VOCs occur naturally in the environment, while others are manmade or both [1]. VOCs are detected in many aquifers across the U.S. and their presence in drinking water may pose a risk to human health because of their potential carcinogenicity. VOCs are quickly eliminated from the body (half-life 1-9 hours[2-6]). VOCs can be exhaled unchanged, but a portion can be bioactivated to form reactive metabolites. From human and animal studies, certain VOCs have been associated with a wide range of cancers including liver, kidney, immune, neurological, and reproductive, as well as developmental toxicity. There is limited human data supporting associations between VOC exposures and breast cancer [2-11], but animal evidence has been reported.

Breast cancer in women is the most commonly diagnosed form of cancer and the second highest killer of women, after lung cancer [12]. In contrast, male breast cancer is exceedingly rare yet incidence rates in male breast cancer are rising [12]. A “hot spot” of male breast cancer is developing around the Marine Corp Base, Camp Lejeune. Over 80 men
who developed breast cancer in the last 10 years with residential ties to Camp Lejeune believe VOC exposure through contaminated well water was an important risk factor in their breast cancer development [13]. Male breast cancer is understudied in animal models. The male rat and mouse mammary gland have been demonstrated to be sensitive to EDCs [14, 15], and further testing of VOCs during windows of susceptibility in the mammary gland are needed to reveal if there is a potential link between male breast cancer and VOC exposure.

As described in Chapter 1, the mammary gland functions to provide nutritional sustenance to offspring and the organ continues to develop throughout life. The mammary gland undergoes its first period of rapid growth during embryogenesis. It continues to further develop after birth; during puberty and pregnancy. Normal mammary gland development involves the communication between mammary stromal cells and epithelium to direct normal mammary ductal tree elongation and branching. Following lactation and during menopause, involution occurs and the mammary gland is remodeled through apoptosis.

During the pubertal expansion of the mammary gland, terminal end buds (TEBs) proliferate as ductal elongation and branching occurs. TEBs are the highly proliferative end units, where most of the growth of the mammary epithelium takes place and are consequently highly susceptible to carcinogenic insults [16]. Once the mammary gland has reached the edge of the fat pad or encounters other glands, the TEBs differentiate and mature into terminal ends. TEBs are abundant during puberty and differentiate as the mammary gland matures. TEBs are particularly sensitive to carcinogens and when they are present in the gland, this represents a window of susceptibility for the mammary gland. Chemically-induced mammary tumorigenesis with the mammary-specific carcinogen, 7, 12-dimethylbenz[a]anthracene (DMBA) occurs primarily in the epithelium of TEB and the
administration of DMBA to rodents during puberty induces the largest number of transformed TEBs, resulting in an increased tumor burden[16-18].

The Harlan Sprague Dawley (HSD) mammary gland atlas in Chapter 2 characterized male and female tissue development and gave examples of the early morphological changes following prenatal EDC exposure that may occur. Studies testing EDCs, like Bisphenol A, have found altered mammary gland maturation rates, enhanced ductal growth and increased susceptibility to carcinogenesis following DMBA-administration [17, 19-23]. Further, in Chapter 3, I determined that there is transplacental and potentially lactational exposure to pups following a Camp Lejeune relevant drinking water exposure to VOCs in the HSD rat. The present studies were designed to evaluate the effects of five VOCs, as a mixture, on mammary gland development and tumor susceptibility in male and female rats following gestation early life exposure. Of the five VOCs tested in this study, PERC and TCE are potentially EDCs. PERC exposure has been associated with menstrual disorders and TCE may reduce FSH and testosterone levels in men [24, 25]. Furthermore, benzene and vinyl chloride have been shown to cause mammary tumor development in female rodents when delivered at high doses over their adult life [2,5]. In this study, timed-pregnant HSD rats were exposed to the same mixture of chemicals as in Chapter 3; 0, 5X and 10X, in the drinking water from GD 12-PND 48. 1X concentrations of VOCs is the level measured in the well water at Camp Lejeune[26]. Mammary glands were collected at PND 23 and 48 and evaluated for growth patterns. A subset of offspring were dosed with DMBA on PND 30 to initiate mammary gland carcinogenesis and assess the ability of VOCs to sensitize mammary glands to a carcinogen.
Materials and Methods

Animals

Time pregnant HSD rats, obtained from Harlan Laboratories (Indianapolis, IN), arrived on GD 10 (GD 0 is defined as plug positive) and allowed to acclimate for 2 days. Pregnant dams were housed individually in polypropylene cages with bedding and nestlets and switched to a rodent phytoestrogen-reduced diet on arrival (Zeigler; Madison, WI) and received chow ad libitum. Animal facilities were maintained on a 12:12 h light-dark cycle at 20°C–23°C and 40–50% relative humidity. All animal studies were approved by the NIEHS Animal Care and Use Committee.

Chemicals

VOC drinking water solutions were prepared as in Chapter 3. Ampules (1 mL) containing standard mixture of benzene, PERC, TCE and t-1,2-DCE were purchased by the Centers for Disease Control and Prevention (CDC) from O2si (Charleston, SC) and VC (2000 µg/ml) was purchased from Sigma Aldrich (Steinhiem, Switzerland). VOC drinking water solutions were prepared by spiking 38.85 µl of the standard and 312.5 µl of VC into 500 ml reverse osmosis/deionized water resulting in 10 µg/ml of benzene, 75 µg/ml of PERC, 10 µg/ml of TCE, 20 µg/ml of t-1,2-DCE and 1.25 µg/ml of VC for the “50X” concentration. Serial dilutions were made for “10X” and “5X” concentrations (Table 3.1). 500 ml glass drinking water bottles with metal sippers, filled with dosed drinking water, were given to animals to drink ad libitum. Pilot experiments showed that VOC off-gassing depends more on the amount of head space in the water bottle than the length of time since freshly mixed. The drinking water bottles were changed at least every other day to ensure no
more than 25% of the water was consumed, to reduce the changes in VOC water concentrations.

**Experimental Design**

Two studies were conducted to determine the effects of a mixture of VOC on body burden.

**Early mammary gland effects study:** Time-pregnant HSD rats (60 dams) arrived at NIEHS on gestation day (GD) 10 and were divided into 3 treatment groups, control (n=15), 5X (n=15) and 10X (n=30) of equal average body weight. Seven dams were not pregnant and were removed from the study (two from control, one from 5X and four from 10X). Each dam and subsequent pups were given treated water to drink ad libitum until sacrifice. At PND 4 pups were culled and litters were equalized to five male and five female pups.

**Carcinogen-induced mammary cancer study:** A subset of male and female pups (n=52-56 per treatment) from each group of the early mammary gland effects study were reserved for DMBA dosing. An additional 52 10X VOC offspring were not given DMBA, but were otherwise treated similar to DMBA treated animals. DMBA (30 mg/kg body weight) was given at PND 30 to all remaining dose groups (Control+DMBA, 5X+DMBA and 10x+DMBA) via oral gavage in sesame oil (5 mL/kg body weight). Pups continued to drink treated water until PND 48 and then were switched to tap water. Pups underwent weekly mammary gland palpations and tumor latency and size were recorded by gland, size and date. Animals were sacrificed about 30 weeks after birth and 26 weeks after DMBA administration.
Necropsy

Early mammary gland effects study: 12 pups per sex per dose group were sacrificed on PND 23 and 48 by CO₂ asphyxiation. Terminal cardiac puncture was performed and blood was collected from the left ventricle for serum and blood smear. Whole body, liver and spleen weights were recorded. One set of 4\textsuperscript{th} and 5\textsuperscript{th} mammary glands were removed for whole mount analysis and the contralateral set was collected into histology cassettes and fixed in formalin for immunohistochemistry (IHC) processing. Sections of liver, spleen, thymus and femur were collected into histology cassettes and fixed in formalin for histology evaluations. A section of liver and mammary gland were flash frozen for RNA analysis. The pathology of the liver, spleen, bone and thymus will not be discussed here as they were part of another project.

Carcinogen-induced mammary cancer study: Four offspring (two males and two females) per dam from each dose group (n=52, 56, 52, and 52 from control+DMBA, 5X+DMBA, 10X+DMBA and 10X-DMBA) were sacrificed at 30 weeks after birth by CO₂ asphyxiation. Body weights were recorded and blood was collected by terminal cardiac puncture. All organs were examined for lesions and mammary glands were removed for histology, along with any other apparent masses. Mammary tumors were measured and size recorded. Livers were collected and weighed. A portion of liver and mammary gland were snap-frozen in a cryo vial for future studies. The following additional target tissues were collected and fixed in 10% formalin for histology: liver, spleen, lung (infused with formalin), thymus, right and left kidney, adrenal, submandibular lymph nodes, uterus and ovaries. Again, the effects of the VOCs on these tissues are not reported here as they are part of another project.
**Mammary Gland Preparations**

Mammary glands whole mounts were prepared and stained with carmine alum as described in [27]. Images of whole mounts were evaluated on a Leica DM2000 or Z16 APO with a Leica DFC295 Camera (Leica Microsystems, Frankfurt, Germany). Using the evaluation criteria specified in Davis and Fenton (2013) [28], a qualitative developmental score was given. A score of 4 represented a gland that was most well developed, while a score of 1 represented few of the necessary criteria needed. Generally, the criteria for a HSD PND 23 female mammary gland to receive a 4 included: 1) growth of the 4th gland past the lymph node, 2) 4th and 5th glands had grown together, 3) generous branching buds and TEBs on the ducts and 4) lobule formation started throughout the gland. The criteria for a HSD PND 48 female mammary gland to receive a 4 include: 1) few to no TEBs present, 2) growth to the edges of the fat pad, 3) very dense branching and 4) multi-unit lobules present. HSD male rats had similar criteria as the females with the exception of criteria regarding the 5th gland. Control male glands were similar to control female glands at PND 23 and were slightly more differentiated than female glands at PND 48. The 5th gland in 50% of the males never formed, in both treated and control rats. Mammary gland assessments were based on the development of the 4th gland. Scorers reviewed controls in each age/sex group prior to sorting the entire set of slides by score. Slides within a score were compared once segregated for uniformity of scoring, before reporting scored for individual slides. Scoring was performed by two reviewers without knowledge of treatment, and the two scores were averaged. Mean scores for treatment groups were calculated for each time point and analyzed for statistical significance.
**ERα and PR immunohistochemistry**

Normal mammary tissue and tumor sections were deparaffinized and rehydrated through a series of alcohols to 1X PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Heat induced antigen retrieval as performed using citrate buffer pH 6.0 (Biocare Medical, Concord, CA) at 120 °C for 5 minutes in a decloaking chamber (Biocare Medical). The sections were incubated with 10% normal horse serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 20 minutes. Endogenous biotin was quenched with the Avidin-Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). Sections were incubated with either anti-estrogen receptor alpha antibody at a 1:50 dilution (Beckman Coulter, Brea, CA; catalog # IM1545) or anti-progesterone receptor antibody at a 1:150 dilution (Beckman Coulter, Brea, CA; catalog # IM1546). For a negative control, sections were incubated with mouse IgG1 isotype control serum in place of the primary antibody (BD Biosciences, San Diego, CA) for 60 minutes at 1:50 dilution. Sections were incubated with the secondary antibody, horse anti-mouse secondary antibody (Vector Laboratories) for 30 minutes at 1:1000 dilution and then labeled (Vector R.T.U. Vectastain Kit, Vector Laboratories) for 30 minutes. Antigen-antibody complex was visualized following a 6 minute incubation with 3, 3' diaminobenzidine (Dako Corporation, Carpinteria, CA). The sections were counterstained with modified Harris hematoxylin, dehydrated, cleared and coverslipped with Permount.

**Statistical Analysis**

Unpaired, two-tailed, equal variance t tests, Cochran-Armitage trend test and log-rank (Mantel-Cox) test of survival curves were performed in GraphPad Prism 6.0. The threshold for statistical significance was set at p≤ 0.05.
Results

**Developmental VOC exposure alters body weights and relative liver weights**

Body weights were recorded on days of necropsy as an indicator of toxicity in female and male offspring. At PND 30, male offspring in the 10X exposure group demonstrated a transient 6% body weight increase over controls. At PND 48, females and males in the 10X exposure group were 6% and 5%, respectively, lighter than controls. The reason for this shift in body weight is unknown, but could be due to decreased food or water consumption in those dose groups. Water consumption was not recorded in this study, as a pilot study did not show decreased VOC-laced water consumption. Absolute and relative (liver:body weight) liver weights were significantly decreased in both females and males dosed with 10X VOC at PND 48. Body and liver weights can be found in Table 4.1 and 4.2.

**Developmental VOC exposure leads to aberrant mammary growth**

The focus of this study was mammary gland development and growth in VOC-exposed rats and the potential mammary tumor risk following carcinogenic induction. Mammary glands of VOC-exposed females and males (late gestation through puberty) displayed characteristics of accelerated development at weaning (PND 23) and peri-pubertal (PND 48) time-points. As an initial evaluation point, carmine stained whole mounts were evaluated for developmental growth patterns in comparison with controls. Mammary glands were assessed a qualitative developmental score based on: longitudinal growth, lateral growth, presence of terminal end buds (TEBS), number of terminal ends, branching density and bud formation separately based on age and sex [28]. Male mammary gland assessment was more difficult than the females due to a male specific phenomenon where the 5th gland was missing entirely in the fat pad. In this study, 52% of males were missing the 5th
mammary gland. This effect is independent of treatment and to our knowledge is an HSD-specific issue. VOC exposure resulted in higher developmental scores in the males at PND 23 and PND 48 and females at PND 48 in both VOC exposure groups, compared to controls. Developmental scores cannot be compared across time points or sexes, as the criteria for developmental assessment shifts between time points and the sexes. Developmental scores for glands can be found in Table 4.3. Mammary glands of VOC-exposed rats at weaning (PND 23) appeared larger in size, had more terminal end buds (TEBs), increased branching, and increased epithelial growth in both males and females at both 5X and 10X concentrations (p=0.03 and 0.03 respectively). At PND 48, all dosed mammary glands had increased side branching density, bud density and decreased number of TEBs compared to controls. Mammary glands of both 5X and 10X exposed females (p= 0.0004 and < 0.0001 for 5X and 10X dosed females, respectively) and males (p= 0.02 and 0.0002 for 5X and 10X dosed males, respectively) were significantly more developed than the controls (Fig. 4.1 and Fig. 4.2).

**VOC exposure does not change sex hormone receptor population**

Immunohistochemical (IHC) staining of mammary gland sections for ERα, PR and AR were evaluated in both male and female glands. To quantify the levels, sections were evaluated for epithelial nuclear expression and given a Quickscore, as described in Chapter 2 [29]. As seen in Chapter 2, ERα and AR expression in the HSD rat showed significant cytoplasmic staining and made evaluation of nuclear staining difficult. PR was not present in male mammary glands at any time point. There were no differences in androgen, estrogen or progesterone receptor expression in mammary epithelium at PND 23 or 48 (data not shown).
**VOC and overall non-mammary gland effects**

At necropsy, there were no significant body weight differences between DMBA-treated control (317g±9), 5X (302g±8) or 10X (308g±6) females. There was a significant body weight difference between DMBA-treated and DMBA-untreated 10X (290g±4) females (p=0.01), which may have been due to the tumor burden in DMBA-treated females. DMBA-treated 5X(491g±6) and 10X(481g±11) males has a statistically significant lowered body weight than the DMBA-treated control (513g±8) males (p=0.04 and 0.03, respectively). There was no difference between the DMBA-treated and DMBA-untreated 10X (494g±8) males. There were a few tumors that developed outside of the mammary gland and included: 1 hemangioma (control male), 1 nephroblastoma (control female), 1 skeletal muscle sarcoma (control female), 1 squamous cell carcinoma (10X female) and 9 Zymbal’s gland tumors (3-control males, 1-control female, 1-5X male, 1-5X female, 2-10X males and 1-10X female) (data not shown).

**VOC exposure was associated with an increased incidence of adenocarcinoma arising in fibroadenoma.**

In this study, mammary tumors began to appear 9 weeks after carcinogen administration. Nine control female offspring, five female offspring in the 5X exposure group, five female offspring in the 10X exposure group and five male offspring in the 10X exposure group, that were also treated with DMBA, died due to mammary gland tumor burden during the 26 weeks post-carcinogen. Two additional males were found dead for unknown reasons (no diagnosis; hemorrhagic lungs) before the end of the study. There were no clinical findings of toxicity or tumor burden in any tissue in the males and females.
exposed to 10X VOC and not treated with DMBA (data not shown). For ease of description, all further discussion will focus only on animals that received VOC exposure and DMBA.

Female and male rat mammary tumor incidences are listed in Table 4.4. Fig. 4.3 shows a representative image of the most common tumor types in this study: fibroadenoma (Fig. 4.3A-B), adenocarcinoma (Fig. 4.3C-D) and adenocarcinoma arising in fibroadenoma (Fig. 4.3E-F). Mammary tumor incidences found in females and males that died before the end of the study are listed in Table 4.5 and the tumor incidences of female and male survivors to necropsy in Table 4.6. Overall, 92% of control females and 27% of control males developed DMBA-induced mammary tumors by 30 weeks of age in this study. Historical data on spontaneous tumor development in HSD rats reported 90% of females and 5% of males developed some type of mammary tumor by 104 weeks [30]. In the female rats in the current study, 42% in the control dose, 41% in the 5X dose and 50% in the 10X dose developed DMBA-induced malignant (“adenocarcinoma”, “adenocarcinoma, multiple” or “adenocarcinoma arising in fibroadenoma”) mammary tumors. 0%, 0% and 8% of control, 5X and 10X dosed males developed malignant tumors, respectively. Of the spontaneous tumors reported at 104 weeks of age, 15% of the females (12% adenocarcinomas and 3% adenocarcinomas arising in fibroadenomas) and none of the males developed malignant tumors [30]. There was no increase in overall tumor incidence due to VOC exposure in the females or males. An unusual mammary lesion was found in the male and female rats; an adenocarcinoma arising within a fibroadenoma. There was a dose dependent trend of increasing incidence of adenocarcinoma arising in a fibroadenoma in the female rats, however the difference in incidence between 10X vs control did not reach significance. There
were no statistical increases or trends in the male rats, however two incidences of adenocarcinomas in males were discovered in the 10X dose group.

**VOC exposure potentially shifts tumor latency in females.**

The latency to tumor formation is shown in Fig. 4.4. Rats underwent weekly mammary palpations where approximate size, location and date were recorded. There was a shift towards a decreased tumor latency in the 5X and 10X females that did not reach significance. There was no significant difference between control and treated males on overall time to tumor formation.

**VOC exposure does not increase multiplicity of tumor formation.**

In the animals that formed tumors, the number of tumors per animal was evaluated. Overall, mammary tumor multiplicity was not affected by VOC exposure in male or female rats (Fig. 4.5A-B). Of the rats that developed tumors, females had an average of 5-6 tumors per animal and males had an average of 2 tumors per animal. Of the females that developed benign tumors (“fibroadenoma”, “fibroadenoma, multiple”, “fibroadenoma with atypia”, or “fibroma”) an average of 4-6 tumors developed per animals (Fig. 4.5C). Of the females that developed malignant tumors, there was an average of 2 tumors per animals (Fig. 4.5D). In the control group, 46% of the female offspring that developed tumors had both fibroadenomas and adenocarcinomas. In the VOC treated groups, 45% and 55% of the female offspring in the 5X and 10X exposure group, respectively, developed both fibroadenomas and adenocarcinomas.

**Immunohistochemistry of malignant tumors for ER and PR staining**

A subset (n=28) of adenocarcinomas and adenocarcinomas arising in fibroadenoma were immunostained for ERα and PR. 62% of tumors from female rats were ER-negative and
PR-positive (Fig. 4.6A-B) and 38% were ER-positive and PR-positive (Fig. 4.6C-D). The two malignant tumors from male rats (one adenocarcinoma and one adenocarcinoma arising from fibroadenoma) were both ER-negative and PR-negative (Fig. 4.6E-F). All of the female adenocarcinomas outside of fibroadenomas (n=11) were ER-negative while 67% (n=15) of female adenocarcinomas arising in fibroadenoma sampled were ER-positive.
Discussion

The present studies demonstrate that exposure to VOCs during mammary gland development (GD 12- PND 48) at drinking water doses (and no inhalation exposure) only 5 or 10 times higher than concentrations found in domestic well water are sufficient to produce accelerated mammary gland development in HSD rats. The highest dose of VOCs (10X) transiently lowered body weight and relative liver weights of both males and females at PND 48, yet accelerated mammary gland development. Developmental exposure to the lower concentrations of VOCs resulted in higher mammary gland developmental scores compared to controls at perinatal (PND 23; male only) and peripubertal (PND 48; male and female) time-points. These data suggest that prenatal exposure to VOC may alter mammary gland development in HSD rats at doses lower than investigated here. Additionally, effects on mammary tissue were observed at doses of VOC (5X) lower than those required to exert an effect on body and liver weight. Those findings implied that in HSD rats, the mammary gland was more sensitive to developmental VOC exposure than body or liver weight loss.

The developmental scores of the mammary glands reveal a surprising VOC-induced acceleration in mammary gland development. The male mammary glands were more sensitive to VOCs at PND 23 than females. Both of the VOC exposed male groups had increased budding and an increased number of TEBs present in mammary glands at PND 23. The male mammary glands at PND 48 also had an overall increased density in both dose groups with some conversion to lobuloalveolar epithelial structures, usually only found in sexually mature male rat mammary glands (as demonstrated in Chapter 2). Exposure to estrogenic compounds have been shown to progress prepubertal development of the mammary gland in males and female rats [14]. None of the VOC in these studies are
considered estrogenic compounds, suggesting at low doses the mixture of VOCs can disrupt endocrine pathways in the mammary gland or an unknown, non-endocrine disrupting mechanisms of action is responsible for these mammary gland perturbations; either way, further mechanistic investigations are required. Previous studies have shown that the male mammary gland is one of the most sensitive markers for EDCs and can be more sensitive than female mammary glands [14, 31,32]. Variations in the female mammary gland, such as number of TEBs, can be high and make detecting changes difficult [14]. However, the male has low variation in TEBs and is more sensitive to estrogenic compounds [14, 31]. These findings suggest that male mammary glands are also more sensitive to non-estrogenic effects than female mammary glands at PND 23. On PND 48 however, male and female mammary glands are both sensitive to mammary gland effects of non-estrogenic chemicals.

An established risk factor for breast cancer is precocious puberty, which includes early age of breast development and menarche [33]. Since 1940, there has been an increased trend toward earlier breast development onset and menarche and a rise in EDCs in the environment may be responsible for this trend [34]. In utero and neonatal exposure to EDCs such as DES and BPA have been shown to permanently change mammary gland development and are associated with an increased risk of developing pre-neoplastic lesions and breast cancer [20, 35-38]. VOCs, when tested at levels much higher than these studies, are not reported to be strong EDCs. Some endocrine disrupting activity in women have been associated with PERC exposure. Lower levels of VOC exposure, may be able to disrupt endocrine pathways in a non-monotonic manner. EDCs have also been shown to induce epigenetic changes and are linked to increased breast cancer risk [20]. EDCs, like TCDD and BPA, have also been found to induce epigenetic change in the germline and promote early-
onset female puberty transgenerationally [39]. Benzene, metabolites of PERC and TCE, and VC alter DNA methylation [40-42]. Further investigation may reveal VOCs-induced epigenetic changes can result in accelerated mammary gland development.

The second half of these studies compares the responses of mammary glands of male and female HSD rats to developmental exposure to 5X and 10X VOCs via drinking water and a “second hit” with DMBA, a mammary specific carcinogen. The data in the present study indicate that exposure during mammary gland development to VOC contaminated drinking water, close to the range of human exposure, did not result in overall significantly increased DMBA-induced mammary tumorigenesis. However, several interesting findings are worth noting. With VOC exposure, there was a significantly increased trend in the incidence of malignant adenocarcinoma arising from fibroadenoma in the female rat. While this is a rare occurrence in humans, it is not an uncommon lesion in rats [43-45]. No previous evaluations of mammary gland for these VOCs have been known to induce these specific mammary tumors in rats during 2 year bioassays [2-5]. An epidemiological study has reported a slightly elevated breast cancer risk for women who were highly exposed to PERC in the drinking water [46]. In the males, all the early deaths due to tumor burden and the only two males that developed adenocarcinomas were in the high VOC exposure group. Malignant tumors have not been reported in 2 year spontaneous tumor studies in male HSD rats [30]. Further testing with low levels of oral and inhalation exposure to a mixture of VOCs may exert a stronger effect on mammary tumor development in both males and females.

Breast cancer is a heterogeneous disease with a growing number of subtypes. Prognosis and treatment for the different subtypes differ and primarily depend on the
expression of hormone receptors ER and PR as well as human epidermal growth factor receptor-2 (HER2). The main subtypes are luminal A (ER-positive and/or PR-positive, HER2-negative), luminal B (ER-positive and/or PR-positive, HER2-positive), basal-like (ER-negative, PR-negative, HER2-negative) and HER2+ (ER-negative, PR-negative, HER2-positive). The percentages of each subtype in The Carolina Breast Cancer Study were 51%-luminal A, 16%-luminal B, 20%-basal-like, 7%-HER2-positive and 6% unclassified [47]. Data from the California Cancer Registry revealed, 82% of the male breast cancers were ER-positive and/or PR-positive, 15% were HER2-positive and 4% were negative for all three (basal-like) [48]. Luminal A has the best prognosis of all the subtypes and basal-like and HER2-positive subtypes have the worst [49]. The ER and PR staining of the malignant tumors treated with vehicle or VOCs demonstrated the tumors from females were either luminal A or B and the two tumors from the males were either basal like or HER2+. 73% of luminal (A and B) tumors in women were ER-positive and PR-positive and only 11% were ER-negative and PR positive [47]. In VOC treated female rats, ER-negative and PR-positive tumors were more common (62%) than ER-positive and PR-positive (38%). All of the female AC sampled were ER-negative while 67% of female AC arising in FA sampled were ER-positive. Since ER-positive/PR-positive cancers are more common in women, the AC arising in FA may be more human relevant. The lack of PR expression in the male malignant tumors may suggest that these are basal-like or HER2+ tumor types. There may be a species-related effect due to the fact that male rat mammary epithelium have no PR present throughout their lives while human males do have PR expression in the mammary glands [50]. BRCA1 mutations are associated with the basal like breast cancer phenotype [51].
Immunohistochemical markers for HER2 will allow for the differentiation of the further tumor subtypes; luminal A or B and basal-like or HER2+.

DMBA is the most commonly used mammary specific tumor initiator used in Sprague Dawley rat model [51]. N-methyl-N-nitrosourea (NMU) is also commonly used in Sprague Dawley or Fischer 344 rat model. DMBA is given by gavage and is metabolized by the liver to its reactive form, while NMU is given by subcutaneous injection and is a direct acting mammary carcinogen. Both compounds yields tumors with similar latency and incidence [51]. DMBA was selected over NMU for its wide use in rats, thus increasing background information that might be gathered in male and female rats, and our laboratory’s familiarity with the compound. NMU induces a higher ratio of adenocarcinomas than DMBA [52]. Concerns about impaired liver metabolism affecting the efficacy of DMBA were alleviated when low levels of VOCs were not seen to affect CYP mRNA levels in Chapter 3 and it was established that DMBA and VOCs are metabolized by varying types of CYPs [2-6,53].

There was an issue with DMBA sensitivity in this study (though NMU would have more than likely had the same issue). The females were highly sensitive to mammary tumor formation. This all may have to do with the timing of administration of the carcinogen. PND 30 was selected to target the life stage when the mammary gland TEBs were most plentiful, as this is a window of susceptibility. DMBA has historically been given on PND 45 in other rat strains to induce mammary gland tumor formation. Because HSD rats mammary glands grow at an accelerated rate naturally and TEBs are at their peak around PND 30 in control animals, we dosed much earlier. Over 92% of the control females developed tumors. A significant increase in total tumor formation in treated animals cannot be detected with that
high of a background rate. When divided into benign and malignant tumor formation, 46% of the control females developed malignant tumors and there was a 10% increase in malignant tumor formation in the 10X females. Perhaps a higher n is needed to statistically see a modest increase in tumor formation. In future studies with this rat strain, DMBA should either be given at a lower dose or a less sensitive time point to avoid a high tumor burden in controls. Although the males are sensitive to disruption of early mammary gland development, they are not sensitive to DMBA initiation of cancer. Only 27% of control males developed tumors and 30% in the high VOC exposure. Also, 2 males developed malignant tumors and they both died early due to their mammary tumor burden. A higher dose of DMBA, spread over time may induce more tumor formation, or a longer period after DMBA dosing may allow for more adenocarcinoma arising from a fibroadenoma to occur.

The rapid development of the mammary gland due to VOC exposure seen early in life could mean the mammary gland is protected from administration of a tumor initiator. The long term harmful effects of accelerated mammary gland development are potentially masked by the short term protection from a shorter window of susceptibility. The TEBs in VOC exposed mammary glands develop and differentiate into terminal ends faster than the controls. When DMBA was administered, the control rat mammary glands have more TEBs present, making them more sensitive to DMBA. Further mechanistic studies on the mode of action of non-estrogenic mammary gland acceleration could reveal alternative risks for VOC exposed mammary glands.
Table 4.1 Female weight indices of offspring from the VOC drinking water exposure study.

<table>
<thead>
<tr>
<th></th>
<th>PND 23 (n)</th>
<th>PND 48 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55.03 ± 0.91 (11)</td>
<td>173.0 ± 1.85 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>54.82 ± 1.28 (12)</td>
<td>169.2 ± 2.22 (11)</td>
</tr>
<tr>
<td>10X</td>
<td>53.07 ± 1.42 (9)</td>
<td><strong>163.4 ± 2.52</strong> (12)</td>
</tr>
<tr>
<td><strong>Absolute Liver Weight (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.45 ± 0.05 (11)</td>
<td>9.57 ± 0.19 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>2.38 ± 0.08 (12)</td>
<td>9.13 ± 0.18 (11)</td>
</tr>
<tr>
<td>10X</td>
<td>2.39 ± 0.08 (9)</td>
<td><strong>8.54 ± 0.20</strong> (12)</td>
</tr>
<tr>
<td><strong>Relative Liver Weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.045 ± 0.001 (11)</td>
<td>0.055 ± 0.001 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>0.043 ± 0.001 (12)</td>
<td>0.054 ± 0.001 (11)</td>
</tr>
<tr>
<td>10X</td>
<td>0.045 ± 0.001 (9)</td>
<td><strong>0.052 ± 0.001</strong> (12)</td>
</tr>
</tbody>
</table>

Note: VOC = volatile organic compound. PND = postnatal day. 5X and 10X = 5-fold and 10-fold water contamination measured at Camp Lejeune [26]. Data presented are mean ± SE; n = 9-12 animals/treatment group. ** indicates p < 0.01 compared with control by Student’s t-test.
Table 4.2 Male weight indices of offspring from the VOC drinking water exposure study.

<table>
<thead>
<tr>
<th></th>
<th>PND 23 (n)</th>
<th>PND 48 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>58.58 ± 1.07 (12)</td>
<td>221.8 ± 2.37 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>57.69 ± 0.81 (12)</td>
<td>216.6 ± 2.32 (12)</td>
</tr>
<tr>
<td>10X</td>
<td>59.07 ± 1.17 (12)</td>
<td><strong>210.0 ± 3.28</strong> (11)</td>
</tr>
<tr>
<td><strong>Absolute Liver Weight (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.67 ± 0.05 (12)</td>
<td>13.11 ± 0.36 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>2.56 ± 0.07 (12)</td>
<td>12.58 ± 0.26 (12)</td>
</tr>
<tr>
<td>10X</td>
<td>2.70 ± 0.08 (12)</td>
<td><strong>11.41 ± 0.24</strong> (11)</td>
</tr>
<tr>
<td><strong>Relative Liver Weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.046 ± 0.001 (12)</td>
<td>0.059 ± 0.001 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>0.044 ± 0.001 (12)</td>
<td>0.058 ± 0.001 (12)</td>
</tr>
<tr>
<td>10X</td>
<td>0.046 ± 0.0004 (12)</td>
<td><strong>0.054 ± 0.001</strong> (11)</td>
</tr>
</tbody>
</table>

Note: VOC= volatile organic compound. PND= postnatal day. 5X and 10X= 5-fold and 10-fold water contamination measured at Camp Lejeune [26]. Data presented are mean ± SE; n = 11-12 animals/treatment group. ** indicates p < 0.01 and *** indicates p < 0.001 compared with control by Student’s t-test.
Table 4.3 Mammary gland developmental scores from VOC drinking water exposure study.

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PND 23 (n)</td>
<td>PND 48 (n)</td>
</tr>
<tr>
<td>Control</td>
<td>2.3 ± 0.2 (12)</td>
<td>1.9 ± 0.2 (12)</td>
</tr>
<tr>
<td></td>
<td>2.1 ± 0.3 (12)</td>
<td>2.3 ± 0.2 (12)</td>
</tr>
<tr>
<td>5X</td>
<td>2.4 ± 0.3 (12)</td>
<td><strong>3.0 ± 0.2</strong> (11)</td>
</tr>
<tr>
<td></td>
<td>2.9 ± 0.2* (12)</td>
<td><strong>3.0 ± 0.1</strong> (12)</td>
</tr>
<tr>
<td>10X</td>
<td>2.9 ± 0.3 (12)</td>
<td><strong>3.2 ± 0.2</strong> (12)</td>
</tr>
<tr>
<td></td>
<td>3.1 ± 0.3* (12)</td>
<td><strong>3.5 ± 0.1</strong> (12)</td>
</tr>
</tbody>
</table>

Note: VOC= volatile organic compound. PND= postnatal day. 5X and 10X= 5-fold and 10-fold water contamination measured at Camp Lejeune [26]. Data presented are mean ± SE; n =11-12 animals/treatment group. * indicates p < 0.05 and ** indicates p < 0.01 compared with vehicle control by Student’s t-test.
Table 4.4 Mammary gland tumor incidences

<table>
<thead>
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<th>Females</th>
<th></th>
<th></th>
<th></th>
<th>Males</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5X</td>
<td>10X</td>
<td>Control</td>
<td>5X</td>
<td>10X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA, Multiple</td>
<td>18</td>
<td>16</td>
<td>14</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA with Atypia</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroma</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>AC</td>
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<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC, multiple</td>
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Note: VOC= volatile organic compound. FA= fibroadenoma. AC=adenocarcinoma. 5X and 10X= 5-fold and 10-fold water contamination measured at Camp Lejeune [26]. Some animals had multiple tumor types and are represented more than once. * indicates a significant trend p < 0.05 by Cochran-Armitage trend test.
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Note: VOC= volatile organic compound. FA= fibroadenoma. AC=adenocarcinoma. 5X and 10X= 5-fold and 10-fold water contamination measured at Camp Lejeune [26]. Some animals had multiple tumor types and are represented more than once.
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Note: VOC= volatile organic compound. FA= fibroadenoma. AC=adenocarcinoma. 5X and 10X= 5-fold and 10-fold water contamination measured at Camp Lejeune [26]. Some animals had multiple tumor types and are represented more than once.
Figure 4.1 Female offspring mammary gland whole mounts in developmental VOC exposure study. (A-C) PND 23 0.8x. (D-F) PND 48 2x. Statistically increased developmental scores were achieved at PND 48, in the dose groups 5X (E) and 10X (F), with increased branching density compared to control (D). n =11-12 animals/treatment group.
Figure 4.2 Male offspring mammary gland whole mounts in developmental VOC exposure study. (A-C) PND 23 0.8x. (D-F) PND 48 2x. Statistically increased developmental scores were achieved at both PND 23 and PND 48, in both the dose groups 5X (B and E) and 10X (C and F), with increased budding branching density compared to controls (A and D), respectively. n = 12 animals/treatment group.
Figure 4.3 Representative images of a fibroadenoma, an adenocarcinoma and an adenocarcinoma arising in a fibroadenoma from DMBA induced tumor study.

Fibroadenomas were variably sized, well defined nodular masses that were often encapsulated and compressed the adjacent mammary tissue (A) 2X (B) 20X. Adenocarcinomas were less circumscribed and often invaded the surrounding adipose tissue. The neoplastic epithelial cells formed densely packed acinar structures that were several layers thick. (C) 2X (D) 40X. Adenocarcinoma arising in fibroadenoma was described as a focally adenocarcinomatous change within a well-defined primary fibroadenoma (E) 2X (F) 10X, (adenocarcinoma, arrow).
Figure 4.4 Latency of palpable tumors in female and male offspring from DMBA induced tumor study. Female (A) and male (B) tumors present in animals at necropsy that were not detected by palpation were censored.
Figure 4.5 Tumor multiplicity in female and male offspring from DMBA induced tumor study. Total tumor multiplicity in tumor bearing females (A) and males (B) in developmental VOC exposure study with DMBA tumor initiation. 100% of tumor bearing control and 5X females and 91% of the tumor bearing 10X female had benign tumors present (C). 46%, 45% and 55% of control, 5X and 10X females, respectively, had both benign and malignant tumors develop and 9% of 10X females had only malignant tumors form (D).
Figure 4.6 Immunohistochemical staining of adenocarcinomas with ERα and PR. Adenocarcinoma from a control female containing negative cells for ERα (A) and positive cells for PR (B) 40x. Adenocarcinoma arising from a fibroadenoma from a control female containing positive cells for ERα (C) and PR (D) 40x. Adenocarcinoma from a 10X male containing negative cells for ERα (E) and PR (F) 40x.
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CHAPTER 5: Conclusions and Perspectives

Novel Findings

In thinking about the question “Could prenatal/neonatal VOC exposure from drinking water potentially increase the susceptibility of men to develop breast cancer?”, several data gaps needed to be investigated. The first was to address data gaps in mammary gland development in the rodent model of choice, the HSD rat. Chapter 2 has produced data that characterized normal and abnormal morphological mammary glands from mammary bud development to sexually mature mammary glands in the male and female HSD rat. The major novel findings are: 1) the differences, but more so the similarities, between the male and female HSD rat mammary gland development and 2) the HSD strain sensitivity to EDCs.

The similarities between male and female rat mammary gland development have not been extensively studied, especially in the HSD rat strain. The most important similarity is in the overall mammary architecture and cellular morphology during early life through puberty time points. From before birth until puberty, male and female mammary glands, and especially the mammary epithelium, are virtually indistinguishable. The mammary gland starts out the same in males and females with mammary bud formation on E15.5. Ductal elongation continues in both sexes for the duration of gestation and at birth, the mammary epithelium have formed ductal trees. Isometric mammary gland development continues during neonatal and prepubertal time points in both sexes, with TEB development by PND 21. Before puberty both male and female mammary epithelium has a tubuloalveolar
morphology. All of these features are in line with male and female breast development in humans.

Despite the many similarities there is still more differences between the male and female rat than there are in humans. Sexual dimorphism begins in the rat on E18.5. An androgen surge in the males induces apoptosis and the connection between the mammary epithelium and the epidermis begins to atrophy in the males [1]. The female gland continues to develop nipples while the males do not. The second instance of sexual dimorphism is during puberty when the female mammary epithelium undergoes exponential growth to fill the fat pad, while growth of the male mammary epithelium abruptly stops before the edges of the fat pad. This characteristic is also shared with humans. The final major difference between males and female rats occurs in the mature mammary glands. Females keep the tubuloalveolar mammary epithelial morphology while the males develop a lobuloalveolar morphology. This is the most important difference between male rats and humans. Human male mammary epithelium is structurally similar to females at all ages but is just considered a rudimentary form of the female breast [2].

Prenatal EDC exposures can adversely affect mammary gland development, impact lactation and ultimately may be carcinogenic [3,4]. To address the HSD strain sensitivity to EDCs, diethylstilbestrol (DES) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were used in hopes of demonstrating accelerated and delayed mammary gland development. In other rat strains, prenatal exposure to DES and TCDD have been shown to accelerate or delay, respectively, mammary gland development and increased risk of mammary tumor formation later in life [5-9]. In the HSD rat, prenatal TCDD treatment also delayed mammary gland development in both the male and female offspring. The mammary glands of females treated
with TCDD never recovered from the developmental delays, as evidenced in mammary whole mounts, suggesting a potentially permanent effect of this pollutant on the mammary gland. By young adulthood (70 days old), the mammary glands of male TCDD-exposed offspring appeared more similar to controls than they had at earlier life stages. It is possible that because the mammary gland of the male becomes dense very quickly, due to lobuloalveolar development, differences were difficult to discern in the male.

Surprisingly, the DES treated males and females did not have the expected accelerated mammary gland development compared to the controls. The HSD rat has an already accelerated mammary gland development when compared to other strains such as the Long Evans (LE) or the Charles River Sprague Dawley (CRSD) [10-11]. The rate of mammary gland development is not dependent on the animal’s size as previously thought [11]. The HSD rat is significantly smaller than both the LE and CRSD, yet has an accelerated development of the mammary epithelium compared to these other strains. DES has been shown to accelerate mammary gland development in other rat strains [5,12], but failed to affect HSD mammary gland development at the dose tested. Previous studies injected the compound subcutaneously and higher levels of DES were used in weanling rats. In the present study, the dams received the compound by oral gavage during late pregnancy. Lower doses of DES were used to avoid maternal or fetal overt toxicity. The lack of DES acceleration of the mammary gland development in the HSD rat strain may be due to the different dose of DES used, route of exposure, or rate of mammary gland development between rat strains. DES-exposed male rats at PND 70 retain some of the tubuloalveolar morphology found in female and immature male glands. DES treated males may have been feminized, or more likely, the mammary epithelium exhibited a delay in maturation.
compared to the control or TCDD treated animals. Similar exposures with later adulthood
timepoints would clarify this question.

The second data gap that needed to be addressed was how were the half-lives and
body burdens of VOCs affected when given as a mixture of several VOCs in
pregnant/lactating HSD dams and the resulting offspring. The reactive metabolites formed
from the individual components of the VOC mixture are largely dependent on dose and route
of exposure [13]. High dose inhalation studies might not form the same metabolites as a low
dose drinking water studies. Embryogenesis and puberty are windows of susceptibility for
the mammary gland [14]. Most toxicokinetic studies in females are in nulligravid adult
animals and do not capture these sensitive time points. The half-lives of the VOCs in the
blood when given as a mixture at GD 15 were similar to half-lives of each chemicals given as
a single exposure [15-18]. Additionally, the VOC were detected in the mammary gland and
in the fetus, demonstrating that in utero and potentially lactational exposure can occur.

The drinking water body burden study that demonstrated that even with unlimited
access to VOC contaminated drinking water, the dams and offspring never drank enough
volume or consistently enough for the VOC to accumulate or reach a steady state in the
blood. The episodic, low dose nature of the mixture exposure made this portion of the studies
difficult to interpret. The VOCs are eliminated from the rat quickly and most drinking water
consumption occurs at night [19]. Necropsies took place at 9:30-10:00AM meaning the lights
were on for 2.5-3 hours and drinking water consumption would have been reduced at this
time. The amount of water consumed in one sitting and the time from last exposure cannot be
determined. Surprisingly the levels of benzene in the blood of both dams and pups, compared
to the other chemicals in the mixture, were much higher than anticipated. Benzene has been
shown to induce mammary tumors in mice at very high doses in a 2-year bioassay in mice [20]. The data suggests that benzene behaves differently when part of a mixture at low doses and deserves further examination. Both the half-life and drinking water concentrations of benzene and TCE are similar in the VOC mixture, yet the amounts of benzene in the blood are significantly higher than TCE and trans-1,2-DCE. VC could not be detected in the blood, as our collaborators at the Center for Disease Control only had assays developed for urinary assessment of VC. Because of the volatile nature of the chemicals tested, the matrix in which they were measured had to be collected under hermetic conditions and we were unable to perform this type of collection for urine and serum form the same animal. Due to these limitations, the half-life of VC in the pregnant dam and body burden concentrations in the fetus remain unknown.

With the knowledge gained from Chapters 2 and 3, the effects of VOCs on mammary gland development and susceptibility to carcinogens could be addressed. The effects of VOC on mammary gland development has not been previously studied because until recently, the mammary gland was not a mandatory tissue for evaluation in test guidelines studies unless female tumors were visually evident at necropsy [21]. The focus of Chapter 4 was to investigate the effects of a mixture of VOCs on the mammary glands of HSD rats. I hypothesized that early life exposure to this mixture may result in increased altered mammary gland development and ultimately increased tumor development in male rats with secondary exposure to the mammary gland carcinogen, dimethylbenz-a-anthracene (DMBA).

To determine the effects on early mammary gland development, VOC exposure began in utero, before the development of the mammary gland bud and continued until necropsy. This exposure paradigm insured the mammary gland was constantly exposed to
human relevant levels of VOC throughout development, through either transplacental transfer, lactation or ingesting treated drinking water. Just after weaning, male mammary gland development was significantly accelerated at both the low and high VOC concentrations tested. The VOC-exposed females also had mammary development that appeared accelerated compared to the controls, but did not reach significance. At PND 48, peri-pubertal male and female rats have accelerated mammary gland development after both low and high VOC exposures. The mammary glands from VOC-exposed animals were more densely branched and larger in epithelial area. Additionally, the mammary glands of VOC-exposed males appeared to have started converting to a lobuloalveolar morphology at PND 48, an indication that they were prematurely transitioned to a sexually mature gland.

Mammary gland acceleration was an unexpected outcome in this study. Previously in Chapter 2, the HSD rats were not sensitive to mammary gland acceleration by DES. But, the DES insensitivity could be a dose-related issue. While this is a novel finding, it suggests that the already accelerated mammary gland development of HSD rats are susceptible to further mammary gland accelerations and that the 10 μg/kg DES doses during gestation were not enough to accelerate mammary gland development. In trying to avoid maternal and fetal toxicity, the dose was too low to affect mammary gland development in this strain. DES given at higher doses after birth in addition to the gestational exposure would likely induce the accelerated mammary gland development excepted and the HSD strain may be less sensitive to EDCs during gestation alone.

The majority of the VOC-exposed and control rats of both sexes were dosed with DMBA or vehicle at PND 30 to initiate mammary gland tumor development. DMBA was chosen over NMU primarily because it a very commonly used model for mammary gland
tumorigenesis in rats [22]. Typically, this exposure to carcinogen would have been on PND 45 or 50, as reported for other rat strains [6, 24]. However, during the course of these experiments, it came to my attention that mammary gland development occurs earlier in this rat strain, therefore I needed to target a time when control glands might still contain TEBs, the known targets for carcinogen action [24]. The study design was finalized and DMBA had been delivered before we knew that the VOCs caused an even more accelerated breast development than in the controls, modifying even our best efforts to target those cells.

The males and females continued on VOC-laced drinking water exposure until PND 48 and were then switched to water without VOC contamination. The animals were monitored for tumor development and underwent full necropsy 26 weeks after they were dosed with DMBA (about 30 weeks of age). The major findings of this study indicate that: 1) a rarely diagnosed tumor type in the mammary gland seems to be much more common in HSD rats; the adenocarcinoma arising in a fibroadenoma, 2) in females, VOC exposure was associated with a statistically significant increased trend in the incidence of adenocarcinomas arising in a fibroadenoma, 3) two males developed adenocarcinomas and both were in the high VOC concentration group and 4) DMBA induced mammary tumors in this study produced different molecular subtypes.

The increased trend in adenocarcinomas arising in fibroadenoma is an interesting finding since these tumors were typically found in older rats. All the rats that developed adenocarcinomas outside of fibroadenomas died early in this study. Fibroadenomas are considered benign tumors but are shown in the study to potentially convert over time to a malignant phenotype. Suggesting that the fibroadenomas are accumulating additional mutations as the animals aged and inducing an adenocarcinomatous change. There was a
difference between the immunophenotype between the adenocarcinomas and the adenocarcinomas arising in fibroadenomas.

All of the tested female tumors were found to be PR-positive, which makes them luminal-like (either luminal A or B). Only the adenocarcinomas arising in fibroadenomas were both ER-positive and PR-positive. IHC staining of the adenocarcinomas were all ER-negative and PR-positive. These findings suggest that the DMBA-induced mutations for adenocarcinomas can be different from the mutations induced in fibroadenomas. The fibroadenomas may have accumulated additional mutations to become malignant but still had the same of the immunophenotypes of the surrounding fibroadenoma. ER-positive and PR-positive tumors are the most common immunophenotype in women [25]. ER-negative and PR-positive is a rarer immunophenotype in women, but both cancer subtypes, being ER-positive and/or PR-positive, are associated with a greater chance of survival in women [25]. ER-negative and PR-negative female rat tumors were not found in this study or in literature [26]. The two malignant tumors found in males were both ER-negative and PR-negative. This is a rare molecular subtype found in men, and is more commonly seen in individuals that carry the BRCA1 gene [27,28] HER2 staining will further differentiate the molecular subtypes and may reveal that the male rat tumors are the even rarer basal-like subtype. While IHC markers may not indicate that the rat tumors would behave exactly as they would in men and women, they will allow for further rat/human comparisons.

The question of whether or not prenatal/perinatal VOC exposure in drinking water could have made men more susceptible to development breast cancer is inconclusive in these studies. Several limitations could have an impact on this conclusion.


**Limitations and conundrums**

These studies focused on VOC drinking water exposure but has left out arguably the most important exposure route for the men and women at camp Lejeune; inhalation. VOCs off-gas very easily from water when exposed to air and humans are exposed to VOC via inhalation, oral and dermal absorption [15-18,29]. The inhalation chambers we had access to are set up for individual animal exposure to a single chemical exposure. The conundrum we ran into was wanting to have human relevant VOC exposure, meaning VOC exposure from gestation to the end of puberty, and not being about to expose litters to a mixture of VOC at once. The inhalation studies are not equipped to 1) house multiple animals (dam and pups), 2) house pups in general (wire bottom cages), or 3) test exposure to a mixture of chemicals at the same time. Therefore it was decided to go with a drinking water only exposure. While the lack of an inhalation component in these studies is a limitation it may also suggest that the amount of VOCs humans were exposed to at Camp Lejeune could have been higher than the levels of exposure in the rats in these studies. The blood levels of the men and women at Camp Lejeune are unknown and the best that could be done was to as closely recapitulate VOC exposure. The 5X and 10X dose levels were chosen with a safety factor of at least 10 in mind.

There are several limitations in the VOC body burden data. The inability to collect VC under hermetic conditions in the urine means we know nothing about how it behaves in a mixture or in the pregnant rat. Although VC was present at very low concentration in the well water at Camp Lejeune and was likely the result of the natural break down of PERC and TCE, it is a potential mammary gland carcinogen. If the toxicokinetics of VC were affected by the mixture of VOCs in the pregnant rat that is something that was missed in this study.
In trying to recapitulate human exposure data verses a study design with minimal unknowns was a constant challenge in these studies. We ultimately decided to be as human relevant as possible, which meant an ad libitum drinking water exposure body burden study. Some variables that came with an ad libitum drinking water exposure study made the body burden data difficult to assess. The fact that individual water consumption could not be determined due to the group housing of litters made determining an actual daily dose impossible. To get around this, conversion estimates had to be made that were less than ideal. Additional experiments are needed to focus less on human exposures and more on how a mixture of VOCs affect the individual chemical’s toxicokinetics. The second conundrum faced with using a human relevant mixture is that the active mixture component(s) are unknown. Testing the individual compounds or mixtures with fewer chemicals could reveal the active component or that it is the mixture is important. These studies may be important for future VOC assessment but are beyond the scope of this project.

A rodent model for male breast cancer has its limitations. Mice cannot be used since most male mammary epithelium is completely destroyed due to the androgen surge during embryogenesis [30]. The limitation in rats comes from the lobuloalveolar mammary epithelium of mature male rats, which is different compared to female rats and male and female humans. How the differences in mature male mammary epithelium affect the human relevance is unclear. Although the lobuloalveolar development of the male mammary gland makes development human relevant experiments more complex, the mammary glands during gestation and early life are very similar. The rat model for in utero and early life exposures to EDCs can be expected to be more human relevant, and alterations in male and female
mammary gland development early in life could potentially occur in both male and female children as well.

**Impact on science**

Recent changes in the NTP test guidelines protocols have made the HSD rat mammary gland atlas an important, timely and useful document [31,32]. It is anticipated to be included in the NTP’s Non-neoplastic Lesion Atlas online, which is a highly used and a free resource for pathologists around the world. Both NTP pathologists and scientists working on the HSD rat strain will be able to use the mammary gland atlas to better understand normal and chemically altered mammary gland development, differences between species and protein patterns for steroid hormones known to have involvement in mammary gland development. Of particular importance are the comparisons that can be made to other rat strains. Knowing the innate strain differences in mammary gland development will allow for better strain selection for future mammary gland studies. The NTP and other institutions are including mammary gland whole mount production in their standard testing protocols in modified one-generation reproduction studies, reproductive assessment by continuous breeding studies and 2-year bioassays [32]. The challenge has been to incorporate evaluation of those collected glands as one of the major end products. The majority of current studies in the literature are based on strains other than HSD and, to my knowledge, strain differences in mammary gland development have not been previously reported. Differences in ratios of subtype tumor formation will also be useful information in strain selection. The HSD females do have more common, luminal subtype of mammary tumors and demonstrate their human relevance. Other strains may produce a different subtype or different ratios of the different subtypes.
While the harmful effects of individual VOCs on numerous organ systems have been extensively studied, traditional toxicity testing with high dose, single daily oral exposure in adult animals leave large data gaps. Non-monotonic dose response, windows of susceptibility, and the effect of additive, synergistic, or antagonistic chemical interactions in mixtures are all missing from traditional testing. While these studies are not conclusive on the effects of VOCs on mammary gland development and susceptibility to carcinogenesis, they bring to our attention the need to accurately model future rodent studies to human relevant exposures of VOCs and all other potential environmental contaminates.

**Future studies**

The recent change of preferential rat strain in the NTP now is using the HSD strain. This change in strain demands depth in understanding of the HSD mammary gland physiology. Some data gaps currently in the literature on mammary gland development in the HSD rat include: 1) a mechanism for the rapid mammary gland developmental rate of the HSD rat compared to other strains, 2) gene expression differences between the tubuloalveolar epithelium of mature females and immature male and the lobuloalveolar epithelium of mature males and 3) what influences (and how) mammary gland growth and development in males before and after puberty. Understanding the main drivers of the HSD’s accelerated mammary gland development would influence how future mammary gland testing is performed and evaluated. Examination of the major sexual dimorphism between male and female rat mammary glands could not only reveal the mechanism by which the male mammary glands develop compared to females but could discover important similarities and differences to human mammary gland development. An in depth comparison of hormone levels and receptor populations in the male mammary gland may elucidate the reasons for
differences in development and growth before and after puberty. Further studies on the male rat mammary gland could determine if the rat is an appropriate model for male breast cancer. Since radiation exposure increases the incidence of mammary tumor formation in both human and rat males, perhaps differences in adult mammary gland morphology/histology does not affect the human relevancy of male rat tumor formation.

The VOC assessment needs to focus on human relevant mixtures. The body burden data in Chapter 3 suggest potentially interesting toxicokinetics of benzene as a low dose mixture in rats. In revisiting the body burden studies in this project, knowing the average daily dose would be key. Although lactational exposure may be very important to the mammary gland developmental outcomes they make VOC body burden assessment very difficult. Future studies would benefit from singly house animals with water consumption recorded, ideally, in either pregnant animals or weaned offspring, to model sensitive subpopulations. Furthermore, VC in a mixture modeling is still needed. Methods of collecting urine under hermetic conditions are necessary. Anesthesia was not used in these studies avoid for any potential interactions with the VOCs, but may not impact VC analysis in the urine.

When administering a mammary gland carcinogen, timing is key. If the mammary gland is hit with a carcinogen when TEBs are at their peak abundance, tumor formation is highly likely. DMBA has historically been given to female rats at PND 45-50. In Chapter 4, DMBA was administered at PND 30 to male and female rats. The reasoning was that the HSD rat has an accelerated mammary gland development and VOCs further accelerate growth; if DMBA was administering at PND 45, the control rats would be nearing the ends of their window of susceptibility and VOC exposed rats would be even closer to the end. By
administering DMBA at PND 30 we were trying to assure that TEBs would be high in the mammary glands of both controls and VOC exposed. This resulted in nearly 96% of control females developing tumors, and many needing to be sacrificed well before 26 weeks after carcinogen exposure. The 5X and 10X VOC-exposed females had 81% and 92% tumor formation, respectively; the slightly lower tumor burden may be due to the mammary glands being more developmentally accelerated and fewer TEBs were present during DMBA administration. While in the males, there were very few animals with tumor formation by the end of the study. This may be because the male rat had far fewer TEBS than similarly aged females. Furthermore, the stem cells in the TEB are thought to be the eventual target of carcinogens, and it is not known if males have a programmed spike in mammary stem cells during mammary outgrowth as do females. These results reveal that precise DMBA dosing for both males and female are needed for this strain of rat. Future studies should evaluate DMBA exposure for the optimal: 1) amount of carcinogen, 2) timing of administration and 3) length of time after administration is needed to elicit a moderate tumor response, in both HSD males and females. With a moderate tumor formation in control (vs the near 100% rate in this study), testing a chemical’s ability to initiate, promote or protect against cancer development will be easier to determine. Once a chemical is discovered to either delay or accelerate mammary gland development, further modification of carcinogen administration could determine if a chemical is shifting the window of susceptibility or causing a permanent change in the mammary gland that may predispose to or protect from tumor formation later in life.

The effects of developmental exposures to VOCs are not complete with this study. While extensive testing has been performed on these chemicals as single exposures at high
doses, there is a lack of testing using human relevant exposures (low dose and as a mixture) through multiple exposure routes. Further enhancements in inhalation methods testing are needed to explore and determine the best way to effectively and humanely perform inhalation studies on pregnant animals and their resulting offspring. The focus of my studies was on human relevant exposures during critical windows of susceptibility, to best mimic the VOC-exposed families and young Marines living at Camp Lejeune. By only being able to examine the effects of drinking water exposure, these studies are missing the crucial component of inhalation exposure.

Findings from this project emphasize the need for further understanding of rat strain differences, particularly for mammary gland development, and the inclusion of mammary glands and early life exposures into standardized guidelines for toxicity testing. In both male and female rats, there were changes in mammary gland development with VOC exposure, which are not reported to be EDCs themselves, and in the male rats, adenocarcinoma formation only occurred in the high dose VOC-exposed group (although significance was not reached). The percentage of males forming adenocarcinomas in this 30 week study (9%) is much higher than previous spontaneous tumor studies in HSD rats or the rate found in controls in this study (0%). Further testing of human relevant exposures of VOCs, during critical windows of susceptibility are needed to assess hazards and risk for the development of male and female breast cancer. These low dose mixture effects in male and female rat mammary glands, as well as novel blood benzene levels following mixture administration in offspring, should be driving forces for further low dose evaluations of these VOCs.
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