Atrazine and Rat Mammary Gland Development

by

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ABSTRACT

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(Under the direction of Louise M. Ball and Suzanne Fenton)

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, ATR) is used to control growth of broadleaf and grassy weeds. It has been registered with the United States Environmental Protection Agency and is monitored to prevent unsafe levels of this possible endocrine disrupting compound from reaching human and animal populations via contaminated surface or ground water. Life-time feeding studies on ATR have shown an increased incidence and decreased time to tumors in mammary glands (MG) of rats, via its long-term effects on the brain and altered estrous cyclicity. This thesis focuses on changes observed in developing MG of female Long Evans rats (offspring and dam) exposed during late gestation to ATR. It also explores possible confounders of observed effects including body weights, pubertal timing, and serum hormone concentrations.

Dams were gavage dosed with 100 mg/kg ATR during late gestation corresponding to fetal mammary bud development and outgrowth. That exposure caused a delay in puberty and mammary development in female offspring. From cross-fostered litters, it was determined that nursing from an ATR-treated dam delayed both puberty and mammary development. However, a brief transplacental exposure to ATR caused delays only in MG development that persist into adulthood. MG development was most delayed in offspring exposed during fetal mammary epithelial cell proliferation (GD17-19), but it was also

delayed in offspring exposed only by nursing from dams treated during that same time. These results suggested that ATR may reprogram fetal and neonatal MG development.

These findings suggested changes in dam MG development or lactation. MG from ATR-exposed dams were developmentally different from those of controls at early timepoints. ATR and its metabolites were detected in the urine, amniotic fluid, and serum of treated dams at several timepoints post exposure suggesting that ATR metabolites may be available to the developing offspring until at least PND11 when abnormal MG development has been detected in female pups. These studies taken together suggest that ATR can have long-term effects on the MG of female offspring and dams without association of confounders. The effects of this brief exposure are persistent, extending into adulthood and affecting weight gain of future generations via the lactational effects.

DEDICATION

"For I know the plans I have for you, declares the Lord, plans to prosper you and not to harm you, plans to give you hope and a future." Jeremiah 29:11.

Our time on earth is a journey and we meet so many others along the way, some who encourage us by joining in our walk and others who pass in and out along the way. I am so grateful to everyone God allowed to be part of my journey thus far and thankful for your role in my life. You are all special and it is with love that I dedicate this work to you.

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I would like to acknowledge all of my professors and mentors at The North Carolina School of Science and Mathematics, North Carolina Central University (Dr. Byrd, Dr Anderson), Massachusetts Institute of Technology (Dr. Sive, Dr. Bradley), the University of North Carolina at Chapel Hill (Dr. Ball), and the US Environmental Protection Agency (Dr. Fenton, Dr. Hines). This has been a great undertaking, and it would not have been possible without you.

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ATR	Atrazine
BW	Body Weight
d	day(s)
EBM	Environmentally Based Mixture
EGF-R	Epidermal Growth Factor Receptor
GD	Gestation Day(s)
kg	kilogram
MG	Mammary Gland(s)
mg	milligram
PND	Postnatal Day(s)
EPA	United States Environmental Protection Agency

CHAPTER 1

INTRODUCTION TO ATRAZINE

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is a widely used herbicide due to its cost effectiveness, persistence, and versatile application time. It is used primarily on commercial corn, sorghum, sugar cane crops, and fruit crops but can also be used on cotton, Christmas trees, recreational turf (parks and golf courses), and on other crops to control broadleaf weeds and some grassy weeds (Gianessi et al, 1998). Atrazine is broken down by plants, microbes, and animals to form four major metabolites; 2-chloro-4,6-diamino-s-chlorotriazine DACT, hydroxyatrazine ATR-OH, 2-chloro-4-amino-6-(isopropylamino)-s-triazine DEA, and 2-chloro-4-amino-6-(ethylamino)-s-triazine DIA (Wu *et al.*, 1998). The mercapturate forms of atrazine and its metabolites have recently been detected in urine (Norrgran *et al.*, 2006). Atrazine is a pesticide of concern because of detection of it and its metabolites in ground and surface water including those that serve as water sources for humans (Baker, 1998).

Atrazine was registered with the EPA by Geigy in 1958, later to become Ciba-Geigy, Novartis, and now Syngenta. A Registration Standard asking for data to support continued registration of atrazine was issued in 1983 because of concerns regarding carcinogenic risk from drinking and using contaminated surface and ground water (Addendum IRED, 2004). A Data Call-In (DCI) was issued by the EPA in Sept 1990 for more data about atrazine. Along with other measures, Ciba-Geigy reduced application rates for corn and sorghum,

deleted some crop uses, and classified atrazine-containing products as Restricted Use Pesticides in 1990 in response to groundwater contamination concerns (Addendum IRED, 2004). In 1991, the Office of Water set a Maximum Contaminate Level (MCL) for atrazine of 3 parts per billion in water under the Safe Drinking Water Act and required frequent monitoring of watersheds. Another DCI was issued in 1992 and more steps were taken to reduce contamination, this time in surface waters. These steps included reducing application for corn and sorghum, expansion of restricted use, and increased setback requirements (Addendum IRED, 2004).

A Special Review of atrazine began in 1994 to examine the cancer risk of people exposed through their occupation, drinking water, or lawn treatments. Two more DCIs were issued in March and October 1995. Additional use restrictions were placed on atrazine in 1996 including no applications within 66 feet of standpipes or in no-till fields unless residue management was being practiced (Addendum IRED, 2004). Atrazine had a revised cancer characterization in 2000, preliminary risk assessments in 2001, and revised risk assessments in 2002 (Atrazine Background, 2003). In the revised cancer characterization of 2000, atrazine was classified as not likely to be carcinogenic to humans. However, its endocrine disrupting capabilities were noted with concern. The Interim Re-registration Eligibility Decision (IRED) was extended by a Consent Decree between the EPA and the Natural Resources Defense Council to January 2003 with revisions due by October 2003 so that amphibian studies and cancer studies could be incorporated into the risk assessments (Addendum IRED, 2004).

The Federal Insecticide, Fungicide, and Rodenticide Act Science Advisory Panel (SAP) met in July 2003 to discuss potential carcinogenic risk and amphibian gonadal

development. Data from epidemiological studies concerning prostate cancer in workers at an atrazine manufacturing plant showed increased prostate cancer rates in those workers. The SAP decided that atrazine was not likely to cause prostate cancer in those workers due to study confounders. Using the SAP decision along with other data, the EPA decided that there is not a potential human cancer risk from atrazine exposure (Revised IRED, 2003). Studies concerning abnormal amphibian development in the presence of atrazine were also discussed at the SAP meeting. There were studies that showed adverse amphibian effects in the presence of atrazine and other studies that showed no effect from atrazine exposure. Due to conflicting results and insufficient data, no change was made to the January IRED. However, it was recommended that more studies be conducted on amphibians (Revised IRED, 2003).

The October IRED was favorable and included an agreement with Syngenta to monitor 40 watersheds during a two year period. If atrazine concentrations exceed the MCL, steps would be taken to remedy the situation. The results from the monitoring studies will determine if further monitoring or mitigation efforts are needed in those watersheds. Rural drinking wells are also being monitored in atrazine use areas. Monitoring costs would be born by atrazine registrants. Syngenta was also required to conduct studies to determine amphibian effects from atrazine exposure to reduce the uncertainty produced by earlier amphibian studies. These studies include assessment of changes in developmental and gonadal morphology, steroid concentration, aromatase activity, and reproductive fitness of amphibian species (Revised IRED, 2003).

In addition to being registered and regulated for use, atrazine is regulated to minimize environmental and health effects. These regulations are set based on data from health effects

studies of animals and epidemiological studies including acute, chronic, teratogenicity, reproduction, and pesticide spray drift studies (Data Requirements, 2003). Safety or uncertainty factors are included to produce exposure levels to protect humans and children from risk from various routes of exposure (Crawford-Brown, 1999).

Drinking water sources, food residues, and occupational exposures are areas of concern for human health. There is very little atrazine residue on foods processed for human consumption and risk from food contamination do not exceed the level of concern for the EPA. In areas of high use, such as the Midwest, atrazine concentrations in drinking water sources exceed the level of concern and so these sources have to be monitored and use of atrazine may be prohibited in these watersheds to lower concentrations. Several studies have been conducted in these areas but the results have been inconclusive.

Recently the EPA finalized the IRED and completed the reregistration process for atrazine (Atrazine Finalization, 2006) prior to completing the cumulative risk assessment for other chlorinated triazine pesticides (Triazine, 2006). The EPA determined that exposure to atrazine, simazine, and their chlorinated metabolites (DEA, DIA, and DACT) through drinking water, food residues, and non-occupational sources was not of concern and will cause no harm to the population (Atrazine Finalization, 2006).

CHAPTER 2

RAT MAMMARY GLAND DEVELOPMENT

The rat mammary gland is a two compartment structure that begins developing during gestation. Growth is continuous throughout life with the majority of growth occurring during puberty under the influence of many hormones and growth factors. When the animal reaches adulthood (sexual maturity), the gland enters a semi-resting state.

The parenchyma and the mesenchyma are the two compartments of the mammary gland. The parenchyma is derived from the ectoderm and commonly called the epithelial compartment. The mesenchyme derives from mesoderm and is known as the stroma which includes the fat pad. The compartments are separated by a basement membrane (Imagawa *et al.*, 1994).

The epithelial compartment consists of the luminal epithelium and the myoepithelium. The luminal epithelium is composed of epithelial cells that line the lumen of the ducts, end buds, alveoli, and lobules (Imagawa *et al.*, 1990, Masso-Welch *et al*., 2000). Columnar and cuboidal epithelial cells line the ductal lumen and flattened epithelial cells line the alveolar lumen (Masso-Welch *et al.*, 2000). Basal epithelial cells of the myoepithelium form a sheath around the ducts and alveoli (Imagawa *et al.*, 1999, Masso-Welch *et al*., 2000). These basal cells also form the basement membrane that separates the epithelial compartment from the stromal compartment (Masso-Welch *et al*., 2000).

The stromal compartment is made of connective tissue and during fetal development, consists as two separate sections. One section is formed from fibroblasts while the other is formed of fat pad precursor cells (Borellini and Oka, 1989; Imagawa *et al*., 1990). During fetal mammary gland development a few layers of fibroblasts surround growing mammary epithelium and form the mesenchyme by packing densely together (Sakakura, 1987). The fat pad precursor cells derive from fibroblast-like mammary stromal cells (Masso-Welch *et al*, 2000) and form the fat pad by differentiating into white fat tissue after birth (Sakakura, 1987) which is incorporated into the stroma. The connective tissue at the edge of the fat pad forms a boundary to which the mammary epithelial cells grow up to, but not past (Masso-Welch *et al*., 2000).

Cellular Development

Perinatal Development

In the rat, at approximately gestation day (GD) 10, a single-layered ectoderm enlarges. This layer, the mammary streak, extends from the anterior limb bud to the posterior limb bud on both sides of the animal (Sakakura, 1987; Imagawa *et al*., 1994; Hovey *et al*., 2002). Cells migrate along the mammary streak and form six pairs of mammary buds (Imagawa *et al*., 1994) which increase in size due to continued cell migration through GD13 (Hovey *et al.*, 2002). From GD14 to GD16 there is little epithelial growth. However, fibroblasts in the stroma begin to form concentric layers around the epithelial cells. During this timeframe, the overlying epidermis is stimulated to form the nipple (Wysolmerski *et al*., 1998). From GD16-21, the epithelial cells proliferate rapidly forming a sprout or cord (Sakakura, 1987) that grows into the mesenchyme (Imagawa *et al.*, 1990). The cord

canalizes and branches, forming the mammary tree (Sakakura, 1987; Imagawa et al., 1994). In the stroma, lipids accumulate in the fat pad precursor cells (Hovey *et al.*, 2002).

Birth occurs in rats on approximately GD21. Following birth, the mammary gland consists of 15-20 ducts branching off of a primary duct (Hovey *et al*., 2002). The ends of the branching ducts are shaped liked clubs (Figure 1, 2; Imagawa *et al.*, 1994; Hovey *et al.*, 2002). Growth during this period of time is isometric meaning that the epithelium and the mesenchyme grow at the same rate as the body (Daniel and Silberstein, 1987; Borellini and Oka, 1989). This growth is hormone independent (Imagawa *et al*., 1994). During isometric growth, birth to postnatal day (PND) 22, the club shaped ends of the ducts regress but reappear during the peri-pubertal time period (Imagawa *et al*., 1994; Hovey *et al*., 2002).

Peri-pubertal Development

Following PND22, the approximate beginning of the peri-pubertal period, the mammary gland begins to grow allometrically (Figure 3A, B). Allometric growth for mammary development is characterized by mammary growth that occurs at a rate two to three times faster than body weight (Borellini and Oka, 1989; Hovey *et al.*, 2002). The club shaped end buds reappear at this time. The end buds or terminal end buds are clusters of epithelial cells, multiple layers thick (unlike other mammary gland epithelial structures) from which ductal elongation and branching occur (Masso-Welch *et al.*, 2000). Terminal end buds are very proliferative structures that consist of a basal surface and cuboidal epithelial cap cells (Daniel and Silberstein, 1987). Cap cells are a type of stem cell that differentiates into luminal epithelial and myoepithelial cells (Masso-Welch et al., 2000), giving form to ductal structures (Imagawa *et al*., 1994).

During the early stages of puberty, the cells of the terminal end buds undergo mitosis and cell migration to form ductules (Figure 4, 5). Apoptosis of the innermost cells occurs to form the lumen of the ductules (Masso-Welch *et al*., 2000). This ductile elongation occurs until the ducts reach the end of the fat pad (Figure 6; Imagawa *et al.*, 1990).

As puberty ends, the terminal end buds bifurcate to form terminal ductules. The terminal ductules cluster together to form alveolar buds (Masso-Welch *et al*., 2000). With the progression of time, the number of alveolar buds increases and the buds differentiate into lobuloalveolar structures until the animal reaches sexual maturity (Figure 2.6).

Adult Development

After sexual maturity is reached, a cyclic remodeling takes place in the mammary gland that corresponds to the estrous cycle, but the gland is considered to be in a semi-resting state. During the beginning of the cycle, diestrus I and diestrus II, the ductal ends are terminal end ductules. During the middle of the cycle, proestrus, the ends differentiate into alveolar buds. The end of the estrous cycle, estrus, is marked by lobuloalveolar structures and rapid apoptosis of these structures. The terminal end ductules reappear at the beginning of the next cycle (Hovey *et al.*, 2002). This remodeling continues until pregnancy occurs or a constant state of estrus is reached in the aged rat.

When pregnancy occurs, lobules and ducts grow to fill the fat pad. In the early stages of pregnancy, ductal side branching and lobuloalveolar formation takes place. Alveoli increase in number and size to fill the space between the ducts within the gland (Masso-Welch *et al*., 2000). As the epithelial structures grow and develop, the stromal adipose tissue reduces to thin strands (Imagawa *et al.*, 1990, Masso-Welch *et al*., 2000). During the late stages of pregnancy, the alveolar cells fill with lipids and luminal cells fill with a basophilic

substance. The number of blood vessels increases to provide nutrients to the cells of the rapidly growing gland (Imagawa *et al.*, 1990, Masso-Welch *et al*., 2000). During this time, many endogenous substances are being transported to the mammary gland for milk production. At parturition, alveolar cells are filled with lipid and lactose-containing secretory vesicles. Crescent shaped cytoplasm enclosed milk fat globules containing lactose and milk proteins are expelled into the alveolar lumen. Processes of the myoepithelial cells contract and push the contents of the lumen (milk) into draining ducts (Masso-Welch *et al*., 2000).

Hormones and Growth Factors

Mammary gland development occurs through cell differentiation and migration, but this development is dependent upon several hormones and growth factors which are expressed during specific periods of growth in the developing mammary gland, mainly embryonic growth and peri-pubertal/pubertal growth. The majority of hormone and growth factor knowledge comes from the study of transgenic mice, but can be extrapolated to rats.

Parathyroid hormone-related protein (PTHrP) is expressed by mammary myoepithelial cells adjacent to the basement membrane beginning during GD14-16. Wysolmerski and colleagues (1998) showed that the embryos of PTHrP-null mice had normal appearing mammary buds but no primary ducts off the buds. The fat pad of the PTHrP-null embryos was also smaller in size compared to that of control embryos. The control embryos displayed an elongated primary duct extending into the fat pad with initial branches. Closer examination showed degenerating epithelial duct cells in the PTHrP KO mice only in the upper dermis and they noted the absence of nipple sheath development. The primary duct of the control embryos were surrounded by a well-developed nipple sheath (Wysolmerski *et al*., 1998). At birth, the glands of the PTHrP-null mice were characterized

by remnants of degenerating ducts while the control mice had a normal duct system with 15-20 branches elongating into the fat pad.

During the peri-pubertal, pregnancy, and lactation periods many hormones and growth factors are expressed and play a major role in mammary gland development. Ovarian estrogen stimulates ductal growth and stromal proliferation through a paracrine mechanism due to the fact that estrogen receptors are found in stromal fibroblasts and luminal epithelial cells in the end buds, but not cap cells of the end buds (Imagawa *et al.*, 1990; Silberstein, 2001; Hovey *et al.*, 2002). It is also required for lobuloalveolar development (Topper and Freeman, 1980). Progesterone stimulates ductal side branching, terminal end bud formation (Atwood *et al*., 2000), and lobuloalveolar development (Silberstein, 2001). Progesterone receptors are found in half of the cells of the terminal end buds, but not in myoepithelial cells (Atwood *et al*., 2000). Growth hormone stimulates terminal end bud formation and ductal proliferation and prolactin stimulates ductal side branching and terminal end bud formation (Silberstein, 2001; Hovey *et al*., 2002).

Bocchinfuso and colleagues (2000) examined estrogen receptor-α (ERα) knockout mice to determine the role prolactin and estrogen play on mammary gland development. They found that ER-α knockout mammary glands displayed an immature mammary ductal structure and had low circulating serum prolactin levels. With a pituitary graft replacing the source of prolactin, the ER-α knockout mice displayed a ductal network similar to that seen in control mice (Bocchinfuso *et al*., 2000). They also found that exogenous estrogen and progesterone could increase ductal growth and alveolar development in ER-α knockout mice, and that prolactin could induce progesterone levels to enhance mammary growth. Naylor and colleagues (2003) determined that prolactin exhibits its effect via an autocrine/paracrine

mechanism. They showed that prolactin produced by mammary epithelial cells is not responsible for ductal side branching and alveolar bud formation, but that endocrine prolactin is responsible. They determined that cell proliferation in lobuloalveoli was regulated by mammary-produced prolactin (Naylor *et al.*, 2003).

Epidermal growth factor (EGF) stimulates the proliferation of myoepithelial cells (Imagawa *et al*., 1994). Transforming growth factor-β (TGFβ) inhibits mammary epithelial cell growth and appears to regulate ductal patterning through stromal receptors (Silberstein, 2001). Insulin-like growth factor I is required for mammary gland architecture and function, ductal growth, and terminal end bud formation (Imagawa *et al.*, 1994). It is stimulated by growth hormone which enhances its ability to stimulate terminal end bud formation (Kleinberg *et al*., 2000). Insulin-like growth factor II is an effector of prolactin in alveolar development (Hovey *et al.,* 2003). Other growth factors, including hepatocyte growth factor, transforming growth factor-α, fibroblast growth factor, and keratinocyte growth factors, are also involved in mammary gland growth regulation but their mechanisms of action are not fully known.

The role of growth hormone and EGF receptor in the mammary gland was examined by Gallego and others (2001). Growth hormone receptor-null adult mice had retarded ductal outgrowth with limited ductal growth. At a later timepoint, the ducts of these animals filled the fat pad but had limited side branching and thinner ducts than control mice. Mammary glands taken from EGF-receptor null embryos at GD18 had only rudimentary ductal structures (Gallego *et al.*, 2001). Many other studies are ongoing to elucidate the role that the growth factors listed above along with other growth factors, activins, and hormones play in mammary gland development. Mammary gland development is a very complex process

and the current knowledge that we have of the interplay of hormones and other factors necessary for growth is just beginning to be resolved.

Postnatal Day 4 Rat Mammary Gland (Whole Mount)

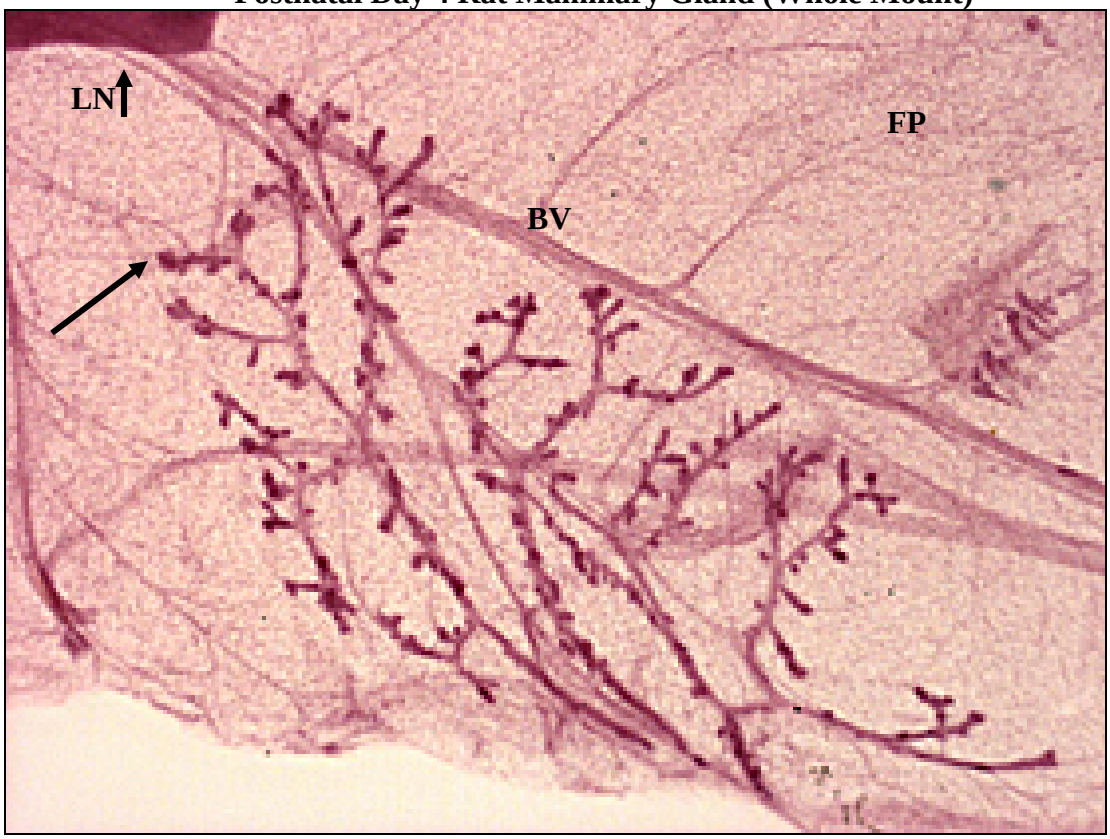


Figure 2.1. Early postnatal mammary gland development. Whole mount of the PND4 rat inguinal mammary gland (4^{th}) stained in carmine alum. This gland has many initial ducts and branches and club-like end buds (arrow) growing toward the lymph node (LN arrow) in the top left corner. Although more difficult to distinguish, blood vessels (BV) and lymphatic vessels are present in the fat pad/stroma (light pink stain, FP).

Histological Section of Postnatal Day 4 Rat Mammary Gland (H&E Stain)

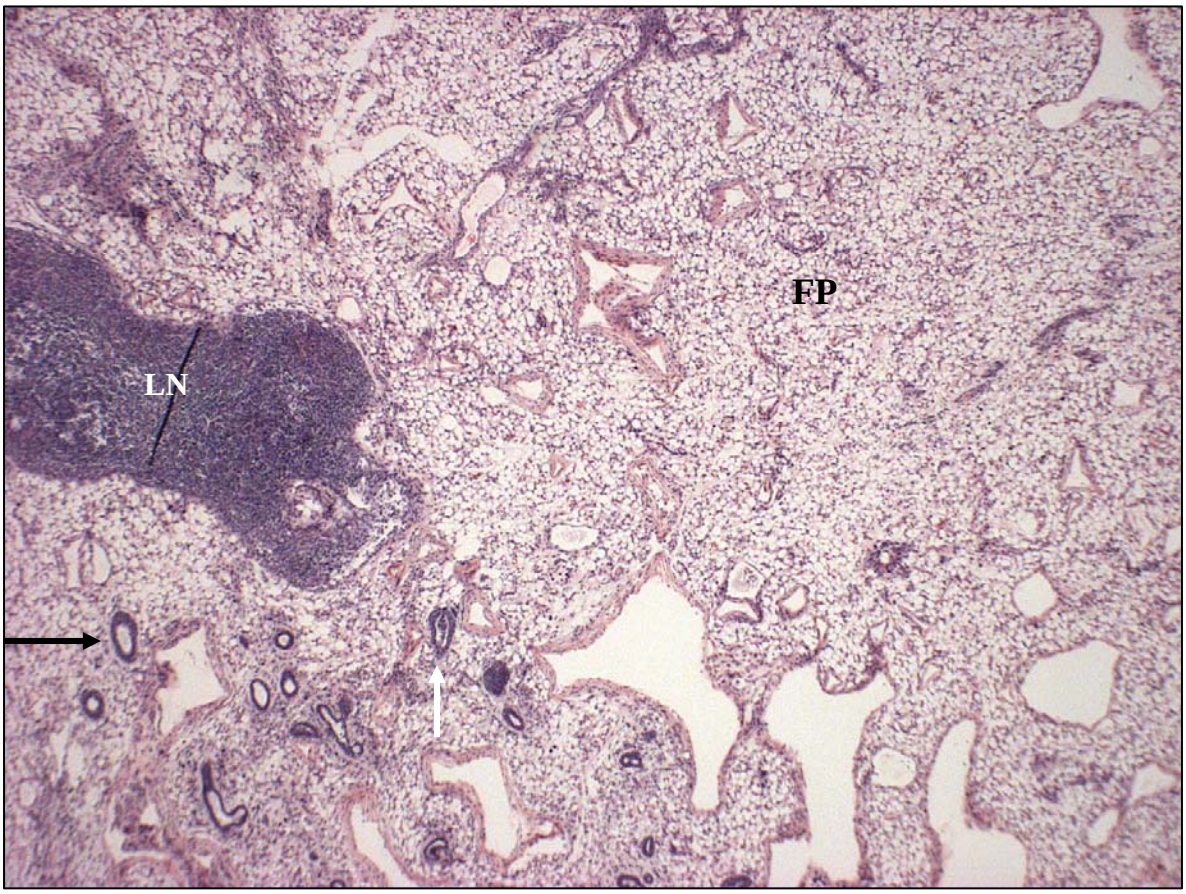


Figure 2.2. H&E stain of the 4th inguinal mammary gland taken from PND4 rat. The small round dark staining structures in the lower left of the slide are the club-like ducts (arrow). Blood vessels are also present within the fat pad (FP). The lymph node (LN) is located in the middle left of the slide.

Postnatal Day 22 & 25 Rat Mammary Gland (Whole Mount)

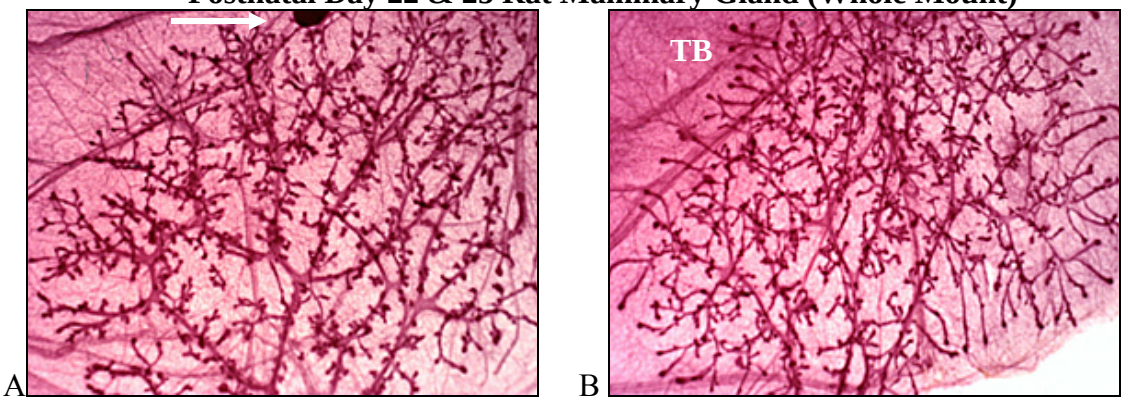


Figure 2.3. Isometric growth of the mammary gland. Whole mount carmine alum stain of the 4th inguinal rat mammary gland prepared on PND22 (A) and PND25 (B). (A) Allometric growth has begun and there is extensive branching of the ducts. Terminal end buds are prominent and ductal growth has reached the lymph node (arrow). (B) In only a short period of time, terminal end buds (TB) are distended and side branches have elongated.

Postnatal Day 33 Rat Mammary Gland (Whole Mount)

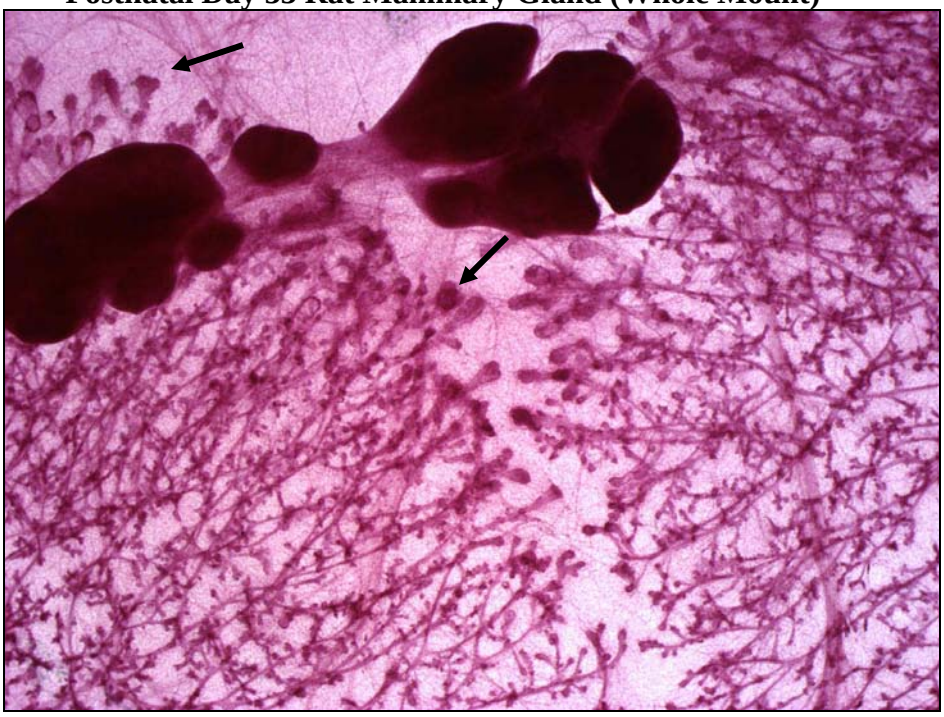


Figure 2.4. Pubertal growth and development of the mammary gland. Whole mount carmine alum stain of the 4th inguinal rat mammary gland prepared on PND33. This is approximately the timepoint when vaginal opening occurs. The gland displays terminal end buds only on the most distal ends (arrows). The fourth and fifth glands are shown growing together. When they reach each other, the terminal end buds will differentiate into alveolar buds and lobules, and rest in a semi-static state.

Histological Section of Postnatal Day 33 Rat Mammary Gland (BrdU Staining)

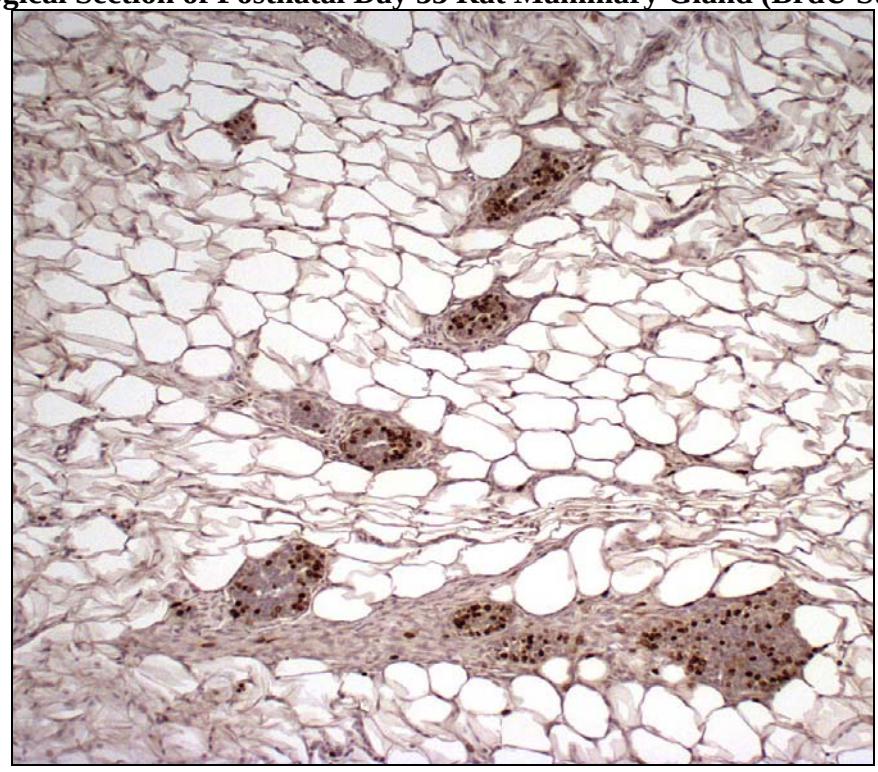


Figure 2.5. Pubertal development of the rat mammary gland. DAB, 3,3'-diamobenzidine, and hemotoxylin stain of a portion of the 4th inguinal rat mammary gland prepared on PND33. BrdU is a cell proliferation marker taken into the DNA during the S-phase of the cell cycle. Dark staining is BrdU labeled cells within ductule structures. The ducts are surrounded by adipose tissue.

Histological Section of Postnatal Day 40 Rat Mammary Gland (BrdU Staining)

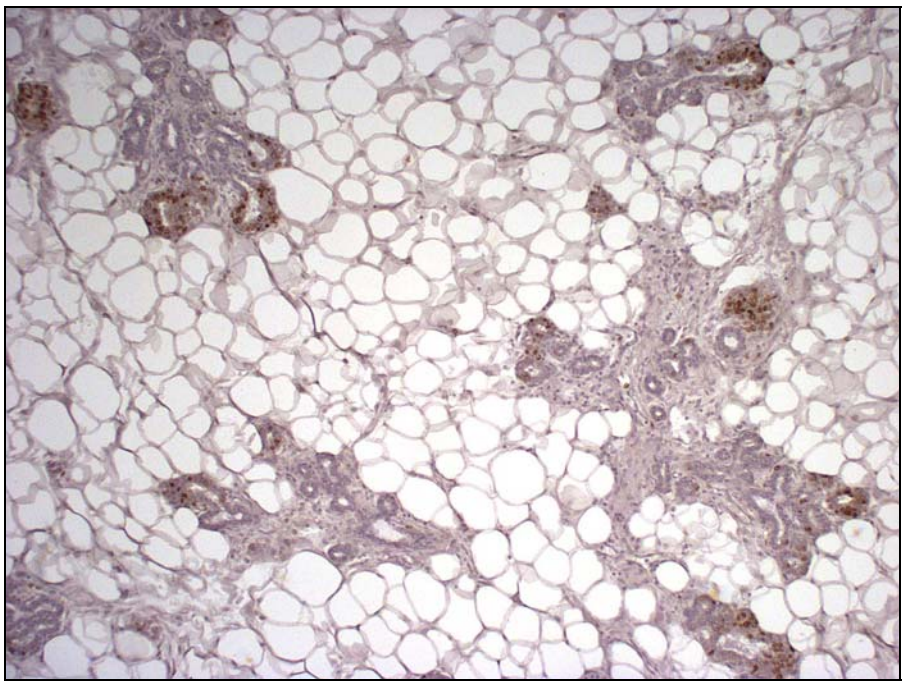


Figure 2.6. Post-pubertal mammary gland development. DAB, 3,3'-diamobenzidine,and hemotoxylin stain of a portion of the 4th inguinal rat mammary gland prepared on PND40. Dark staining is BrdU labeled cells within ductule structures. Fewer structures are labeled compared to Figure 2.5 because cell proliferation is slowing at this time. The animal is nearing the end of puberty and the gland is about to enter the resting stage**.**

CHAPTER 3

MAMMARY GLAND & ENDOCRINE DISRUPTING COMPOUNDS

Endocrine disrupting compounds (EDCs) are chemicals that change endocrine function. They can block or enhance the effect of endogenous hormones through mimicry or direct stimulation or inhibition of the endocrine system (Endocrine Primer). Disturbing the endocrine system during major periods of growth and development can lead to adverse health effects later in life. This is especially true of mammary tissue following EDC exposure during gestation and during the perinatal period.

Several studies have shown that the developing mammary gland is sensitive to toxicant exposure. Fenton and colleagues (Fenton *et al.,* 2002) showed that the mammary glands of female offspring of Long-Evans dams exposed *in utero* to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a lipophilic aromatic hydrocarbon, on GD15 displayed persistently delayed development into adulthood. The mammary gland epithelium of female offspring at several developmental time points had not migrated through the fat pad, lateral branching was reduced, and terminal end-buds were present for extended lengths of time suggesting a critical window of development during which the gland was susceptible to dioxin. Foster *et al.,* (2004) treated Sprague-Dawley dams from gestation day (GD) 9-16 with a mixture consisting of organochlorines, chlorinated benzenes, and metals (Wade *et al.*, 2002) at their individual acceptable daily intake levels, with or without postnatal genistein. Female offspring receiving both the mixture and genistein had increases in female mammary

gland morphological alterations including calcifications, epithelial hyperplasia, and cystic dilation compared to control and mixture groups. Bisphenol A, compound used to manufacture plastic, was shown to alter the development of CD-1 mouse mammary gland when exposed beginning on GD9 and continuing throughout the pregnancy (Markey *et al.*, 2001). Glands removed 1 month after birth from offspring of dams treated with 25 µg/kg showed increased ductal elongation while glands from dams treated with 250 µg/kg showed decreased elongation compared to control glands (possibly due to different signaling components for the low dose effect). By six months of age both groups had significant increases in ductal and alveolar structures compared to control. These studies taken together demonstrate that the fetal mammary glands of rats and mice are sensitive to the effects of environmental agents during the prenatal period of development, a probable critical period of development. The delays in growth and development result in the presence of proliferating structures (terminal end buds) for a longer amount of time. During this time, the mammary gland is more susceptible to 'hits' from carcinogens.

Epidemiological studies concentrating on sexual maturation and growth have been conducted in populations accidentally exposed to endocrine disrupting compounds. Pubertal development was assessed by Blanck *et al*., (2000) in girls exposed to polybrominated biphenyls (PBB) via maternal ingestion, either *in utero* or through breastfeeding. The exposure occurred in 1973 after accidental contamination of Michigan dairy and animal products. They found that females exposed to high estimated levels ($\geq$ 7 parts per billion, ppb) *in utero* and through breastfeeding experienced menarche at an earlier age compared to females breastfed and exposed to low levels or females not breastfed. Breast development in these females was not affected by exposure. Blanck and colleagues (2002) also examined the

growth of these same females. They found no association between height or weight and PBB exposure. The did find that mothers who had higher ($\geq$ 5 ppb) PBB exposure had female children who weighed less than those exposed to low levels of PBB. den Hond *et al.,* (2002) assessed Belgium adolescent populations in two suburbs exposed to polychlorinated aromatic hydrocarbons (PCAHs) and one rural control area. The sources of exposure were a smelter, two waste incinerators, and a crematory. Sixty percent of the population was females, and samples were taken from the adolescent participants. They found that a significant number of females in one of the polluted sites (Wilrijk) had not reached the adult stage of breast development, by a mean age of 17.4 years, and pubic hair was less developed in these females. They also noted that a higher serum concentration of dioxin-like compounds was associated with a lower stage of breast development. Menarche was not different in the three populations. These studies begin to suggest that endocrine disrupting compounds can disrupt puberty and mammary gland development through varying exposure routes, not only in rodents but in the human population as well.

Atrazine (ATR) is a widely used herbicide applied to a variety of crops to control broadleaf and grassy weeds. It is less expensive and persists longer in the soil than alternative herbicides, which leads more farmers to use ATR on their corn, cotton, citrus, and grape crops (Gianessi, 1998). ATR levels higher than the Environmental Protection Agency's (EPA) drinking water standard of 3 µg/L or 3 parts per billion have been detected in surface and groundwater (Baker, 1998).

Stoker and colleagues (1999) found that at least 50 mg/kg ATR completely suppressed the suckling-induced prolactin release in lactating dams treated PND1-4. The dam normally secretes prolactin into the milk for the developing pup which does not begin to

produce prolactin for itself until after PND5 (Hoeffler *et al.*, 2002), and as previously stated, prolactin is needed for adequate mammary gland development during lactation. ATR presumable prevented normal levels of prolactin from getting to the pups resulting in increased incidence of lateral prostate inflammation in adult male offspring. They also suggest that the observed effects could be the result of suppressed immune response due to a transient period of hyperprolactinemia in the male offspring prior to puberty. Other studies examined the effects of single ATR metabolites on puberty in female and male rats (Laws *et al.*, 2003, Stoker *et al.*, 2002). They found that DACT delayed puberty in females and DIA, DEA, and DACT delayed puberty in male rats at relatively high levels. They found the chlorinated metabolites to be as potent as ATR. Cooper *et al.* (2000) showed that ATR specifically inhibits the estrogen-induced surges of luteinizing hormone (LH) and prolactin (PRL) in ovariectomized SD and LE rats and therefore disrupts hypothalamic-pituitary-ovarian activity.

Our early studies of late gestational exposure to high levels of ATR showed delayed mammary gland development in the female offspring. Normal fetal mammary gland development is dependent upon hormones and growth factors directing cellular growth and migration. It is possible that low prolactin levels or low levels or other hormones/growth factors *in utero* or in milk during a critical period of development could alter mammary growth.

CHAPTER 4

ATRAZINE EXPOSURE DELAYS MAMMARY GLAND DEVELOPMENT

Summary

To evaluate the effects of time and duration of exposure to ATR on puberty in LE rats, these studies utilized brief gestational exposures and evaluated the effects on traditional pubertal indicators, as well as mammary gland development, in the female offspring. Early studies suggested that gestational exposure to ATR affects both mammary gland development and VO in female offspring. To evaluate whether these effects of ATR were via similar exposure parameters, offspring were cross-fostered at birth and evaluated for changes in pubertal indicators, reproductive tissue weights, and endocrine hormone status. The exposure parameters were designed to determine if exposure-induced changes were the result of transplacental exposure to ATR (ATR-C), changes in milk composition, (C-ATR), or a combination of the two parameters (ATR-ATR). Additionally, gene expression of local factors, previously suggested to affect mammary gland growth, was evaluated in tissues exposed to ATR under these described routes.

Methods

Animals- Time-pregnant Long Evans rats (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed one per cage and given food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. They were maintained in a room with a 14:10 hour light cycle, 20-24°C and relative humidity of 40-50%. Animals were treated as approved by the National Health and Environmental Effects Research Laboratory, Institutional Animal Care and Use Committee.

Dosing Solution and Procedures- Atrazine (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. Time pregnant rats were treated with 0 (vehicle) or 100 mg atrazine/kg BW by oral gavage in 5 ml/kg dosing volume. The reference dose (100 mg/kg) was chosen due to consistent reproductive endpoint effects observed in previous studies (Stoker et al., 1999, Laws et al., 2000).

Experimental Design- 40 pregnant LE dams were treated on gestational days (GD) 15-19 with vehicle (control) or 100 mg/kg atrazine once daily (N=20 dams/treatment). On postnatal day 1 (day of birth), litters were weighed and approximately half of each litter was cross-fostered with half of another litter from a control (C) or atrazine treated (ATR) dam, creating four exposure parameters (exposed dam-milk source; C-C, ATR-C, C-ATR, ATR-ATR). Pups remaining in the same litter were marked on the tail and/or front right paw with permanent ink. Litters were weighed and equalized to 10 pups on PND4. At weaning, PND22, animals were weighed and separated into unisexual sibling groups, 2-4 female rats per cage. Some females were sacrificed on PND4, 22, 33, 40, and 58. Male pups were used in a separate study unrelated to this one (Appendix B). The data from an exposed dam's

offspring were evaluated with the dam (2 pups/dam or litter) as the experimental unit, with the exception of vaginal smears (see Statistical Analysis).

Vaginal Opening and Cyclicity-Beginning on PND28, females (N>29/group) were evaluated for vaginal opening (VO). The postnatal day of complete VO, and body weight on that day, were recorded. Daily vaginal smears were collected beginning the day following VO for each animal. Smears continued until PND 58. Vaginal smears were read wet for the presence of leukocytes (diestrus), nucleated epithelium (proestrus), or cornified epithelial cell (estrus) to determine cyclicity patterns on an American Optical low-power light microscope. Smear data were plotted in Lotus 123 by assigning the value of 1 to diestrus, 5 to proestrus, and 10 to estrus. The graphs were observed for 4-5 day normal cycles and the number of consecutive normal cycles was recorded for each animal. This was done to evaluate the effect of prenatal atrazine exposure on early cyclicity patterns following VO. Episodes of persistent diestrus or estrus were also recorded and the number of episodes per animal was compared across treatment. Irregular cyclicity was defined as those not presenting at least two consecutive normal cycles between VO and PND 58.

Necropsy-Sufficient female pups were available that on PND58, females in the diestrus stage of their estrous cycle were decapitated, providing N=14 to16 for each of the four cross-fostered groups. Trunk blood was collected and centrifuged for 30 minutes at 3000rpm (4°C) for serum. The pituitary gland, right and left ovaries, and uterus (wet) were removed and weighed. The pituitary gland was frozen at -80°C and the uterus and ovaries were placed in a histology cassette and fixed in 10% buffered formalin.

Mammary Whole Mounts- The 4th and 5th mammary gland were removed, fixed, and stained in carmine as a whole mount as previously described (Fenton *et al.*, 2002) on PNDs 4, 22, 33, 40, and 58 (2-4 pups/dam). Animals on PND 33 and 40 were decapitated and serum obtained for RIAs. Flattened whole mounts were visualized and subjectively scored on a scale of 1-4 (1=poor development/structure and 4=normal development/structure for each age group) by two individual scorers, without knowledge of treatment, within age group (16% coefficient of variation). Mean scores for each age group were computed and analyzed for differences due to treatment. Mammary glands representative of the mean score of the group were photographed on a Leica WILD M420 macroscope.

BrdU Incorporation Assay- On PND 33 and 40, females in diestrus (N=5/exposure parameter) were injected IP with 50mg BrdU/kg BW 3 hours before decapitation. A portion of the 4th mammary gland was fixed in methacarn (6:3:1, v:v:v, methanol, chloroform, and glacial acetic acid). Tissues were processed by Veritas Laboratories, Inc., (Burlington, NC) as stated: 15 min 95% ethanol (EtOH), 3 hr methanol, 4 hr methanol, 1 hr 95% EtOH, 1 hr 100% EtOH, 1 hr xylenes, 1 hr paraffin, 1 hr paraffin, and 3 hr paraffin. The 5µm sections were either stained with hematoxylin and eosin (H&E) or used directly for immunohistochemical detection of BrdU.

Immunohistochemistry- Unstained slides were deparaffinized in xylene (3 changes), hydrated in a series of graded ethanols, and rinsed in distilled water. Sections were then incubated with anti-mouse BrdU (Dako, Carpinteria, CA) 1:40, and biotinylated rabbit anti-mouse IgG (Dako) 1:200, as described (McGinley *et al.*, 2000). Nuclei were stained with 3,3'-diamobenzidine (DAB) (Sigma Chemical) and counterstained with Harris hematoxylin (Fisher Chemical, Fairlawn, NJ) to detect the presence of BrdU.

BrdU Scoring- Slides were visualized on an Olympus BH2 light microscope. For scoring purposes, the total number of epithelial structures per slide was counted. The total number of labeled structures per slide was counted to yield the percent of structures labeled. A score was given to each slide based on the amount of labeling within the labeled structures (1=few cells labeled, 4=majority of cells labeled) and is presented as labeling score.

Radioimmunoassays- Serum luteinizing hormone (LH), prolactin (PRL), and thyroid stimulating hormone (TSH) were analyzed by radioimmunoassay using materials supplied by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases. All assays were run in duplicate with 100 µl of sera per animal. These methods have been previously described in detail (Stoker *et al.,* 2000).

Real Time Polymerase Chain Reaction- The contralateral mammary glands of animals decapitated at PND33 and PND40 were frozen for RNA. Total RNA was extracted from these glands using Tri Reagent as described by the manufacturer (Sigma Chemical), with two chloroform extractions, and dissolved in DEPC-treated H_20. RNA samples were digested with DNAse I (Promega Corporation, Madison, WI) and quantitated using RiboGreen quantitation reagent (Molecular Probes, Inc., Eugene, OR) according to manufacturer's instructions. After incubation with DNAse I, 0.5 µg of random hexamers (Promega Corp.) was added to each RNA sample (N=40 samples), heated to 70° for 5 minutes, incubated on ice, and added to the reverse transcription (RT) reaction. RT reactions were prepared by mixing 4 µl of 5X ImProm-II buffer (Promega Corp.), 2 µl of 10 mM each dNTP, 2.4 µl of 25 mM MgCl, 20U RNasin (Promega Corp.), 1 µl of ImProm-II Reverse Transcriptase and DEPC-treated H_20 (q.s. 10 µl). This RT reaction mix was added to each RNA sample and incubated according to ImProm II manufacturer's instructions. A 100 ng aliquot of each

reaction was then amplified by real-time polymerase chain reaction (PCR) in an iCycler (Bio-Rad Laboratories, Hercules, CA) with gene specific primers and dual-labeled (Fam and Black hole quencher-1) fluorescent probes synthesized by Integrated DNA Technologies (Coralville, IA) for aromatase and epidermal growth factor receptor (EGF-R). Aromatase primers and probe used for PCR were: 5'-TCATTAACGAGAGCCTGCGG-3' F, 5'-TTAACCGGGTAGCCGTCAATC-3' R, and 5'-TGTCGTGGACTTGGTCATGCGCA-3' probe. EFG-R primers and probe used for PCR were: 5'-TGAAGGGAAGTACAGCTTTGGTG-3' F, 5'-CTTCGTAGTAGTCAGGCCC ACAG-3' R, and 5'-TGCCCCCGAAACTACGTGGTGACA-3' probe. The predicted size of the PCR amplified product was 90 bp for aromatase and 109bp for EGFR. Each cDNA was amplified in reactions containing 2 μl of 10mM each dNTP, 5 μl of 10X Thermo buffer (Promega Corp.), 1.2 μl of 10 μM gene-specific reverse primer, 1.2 μl of 10 μM gene-specific forward primer, 0.125 μl of 10 uM dual-labeled probe, 1μl of Taq I polymerase (2.5U Taq, Promega Corp., mixed with 2.5U of Platinum Taq antibody, Invitrogen Corporation, Carlsbad, CA) and 25 mM MgCl (16 μl of 25 mM MgCl for aromatase or 8 μl of 25 mM MgCl for EGFR reactions) in a total reaction volume of 50 μl. PCR cycling conditions were an initial 95° for 3 minutes then multiple cycles of 95° for 15 seconds, 56° for 20 seconds and 72° for 10 seconds. Product size was confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Bands were not detected when reverse transcriptase was omitted, (data not shown) nor when water was the template. Data were reported as threshold cycle and was obtained using manufacturer's recommended settings for iCycler software, version three. Fold differences (induction values) were calculated by determining the differences in threshold cycles for each gene between the control and exposure parameters and log-

transforming the values (exponent of the base 2), due to the exponential nature of PCR (Ginzinger, 2002). Dam means were used as the unit of comparison.

Statistical Analysis- Dam means (vs. litter means, because half litters were crossed) were calculated for body and tissue weights, mammary gland scores, VO day, serum hormones, and real time PCR (threshold and transformed data) and used as the unit of analysis. Means and adjusted means relative to body weight were calculated for organ weights and mammary gland scores. Two-way analysis of variance (ANOVA, Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC) was performed for interaction effects of body weight, and VO day with treatment on mammary gland scores and tissue weights. There were no significant interactions found. Body weights, mammary gland scoring, BrdU scoring, serum hormone concentrations, and PCR data were evaluated for treatment effects within each age group by one-way ANOVA. Analysis of covariance was used to evaluate the effects of treatment on outcomes (mammary gland scores, tissue weights) while adjusting for any effect of body weight. When there was an overall difference between treatment groups, a pair-wise comparison was made between treatment groups and control. Estrous cyclicity data were compared by Mantel-Haenszel analysis (Nonzero Correlation and Row Mean Score test). Significant treatment effects were indicated by $p<0.05$ and specific p-values are indicated.

Results

Dam Weight Gain- Dam weight was recorded throughout the dosing period (GD15-19) and weight gain compared among the groups. Mean body weight of the ATR dams on GD19 (346.7±7g) was reduced 13% when compared to control dams (396.7±7g). Control dams gained an average of 53.3±1.6g during the five-day dosing period and ATR-treated dams gained only 19.3±3.6g during the same time period.

Body Weight, Puberty, and Cyclicity-The offspring of dams treated with ATR and crossed to an ATR dam during lactation were smaller ($p<0.01$) than offspring of all other groups at PND4 (Fig. 1A, 8.2±0.1 ATR-ATR vs. 8.7±0.1 C-C, 8.9±0.2 ATR-C, and 8.8±0.2 C-ATR). While found to be statistically significant, their mean weight was reduced only 5.7% from control animals, which is within normal range. By weaning, PND22, body weights were not significantly different between groups (Figure 4.1B, C-C 54.7±0.8, ATR-C 52.7±0.7, C-ATR 53.1±1.0, and ATR-ATR 53.8±1.0). Furthermore, there was no difference in BW between groups at PND33, 40 (Figure 4.1C, 4.1D), or 58 (not shown).

The female offspring from dams (N>8/exposure parameter) were evaluated for physical signs of puberty, namely, VO and estrous cyclicity. Control animals underwent VO on PND 33±0.4 (Figure 4.2A). A significant delay in VO was seen only in those animals nursing from ATR treated dams (C-ATR 35.4±0.6, ATR-ATR 37.1±0.5, $p<0.02$ vs. C-C). Body weights of these animals on the day of VO were increased significantly in the ATR-ATR, but not the C-ATR groups (Fig. 2B; ATR-ATR $p<0.002$ vs. C-C and ATR-C).

Following VO, estrous cyclicity patterns of these animals were observed until they were necropsied. The number of consecutive normal cycles (4-5 days) per animal were

determined and analyzed according to treatment (Table 4.1). Abnormal cyclicity was designated to groups determined by their smear profiles: persistent estrus (PE), persistent diestrus (PD), or irregular (IR). Animals that were PE and PD exhibited more than 4 continuous days of estrus or diestrus, respectively. Animals marked with IR did not cycle regularly, but could also display PE or PD. Analysis of these data demonstrated no significant differences due to treatment in the number of consecutive normal estrous cycles between VO and PND 58. The number of animals in each treatment group demonstrating PE or PD was also evaluated and no differences were found between treatment groups (data not shown). We found 8% of all animals displayed a period of PE and 17% a period of PD during the first 3 to 4 weeks following VO. We also found that 18% of female offspring did not demonstrate at least two consecutive normal cycles during this peri-pubertal period (consistently irregular).

Mammary Gland Development- In addition to the traditional indicators of rodent puberty, we also evaluated mammary gland development in female offspring. In order to evaluate the effects of atrazine following gestational and/or lactational exposures on mammary gland development in female offspring, whole mount analyses of the fourth and/or fifth mammary gland were compared on PND 4, 22, 33, 40, (Figure 4.3, Table 4.2) and 58 (not shown). Glands from PND4 females were examined to determine if epithelial development of the mammary gland was affected by the ATR exposure parameter. Mammary glands from all ATR exposure parameters were smaller and displayed immature ductal structures compared to glands from C-C animals which showed a normal growth pattern, complete with strong branching. Because the animals in the ATR-ATR group were smaller on average, we evaluated the mammary gland development data using body weight as the covariate and

found the development to be significantly different based on treatment alone ($p<0.001$). This showed that body weight was not causal in the delayed development observed.

At weaning, PND22, control females displayed normal branching with distended terminal ends and abundant lateral branching structures. However, females from the ATR exposure groups displayed sparse branching patterns, fewer terminal ends, and delayed migration through the fat pad. At the peripubertal timepoint, PND33, the glands from the animals that had suckled an ATR treated dam had terminal end buds on all sides and the fourth and fifth glands had not grown together. The glands of C-C females had mostly grown together and only had terminal ends buds remaining on the distal ends. By PND40, the glands from all ATR exposed animals had grown together but had not filled the majority of the fat pad, and still retained large numbers of terminal end buds on both ends compared to C-C animals in which few, if any, terminal end buds remained on the distal ends. By PND58, there were only subtle differences between the groups in mammary gland development or structure. Analysis of the mammary gland scoring at all later time points, using body weight as the covariate, found only exposure parameter-related effects (as indicated in Figure 4.3, Table 4.2).

BrdU incorporation was used to measure the proliferation index (% cells per structure stained) of cells in the rapidly developing mammary gland of animals at PND33 and 40 (McGinley *et al.*, 2000; Table 4.3). Immunohistochemical staining of PND33 gland sections showed no significant differences in staining in animals that had suckled from an ATR treated dam from those who had suckled from a control dam (Table 4.3), even though there were differences detected in the percent of structures labeled that varied from 10-22% higher than C-C. An unusually large variability was seen in the mean % structures labeled, which

could be due to the section of the gland analyzed between animals (long ducts vs. end structures) or the small surges in growth that occur during transition through the estrous cycle. The number of cells labeled, per structure, at PND33 were approximately the same across the groups. However, by PND40, those animals that had suckled from an ATR treated dam had twice the number of cells labeled per structure ($p<0.005$ vs. C-C and ATR-C) than those suckled from a control dam (Table 4.3). These data reinforce the suggestion that final differentiation was delayed in the animals suckling an ATR treated dam.

Gene Expression by RT-PCR- Several hormones and growth factors are known to be involved in mammary gland development. Aromatase, which catalyzes the conversion of androgens to estrone and possibly plays a role in the amount of estrogen found in the breast, is expressed in normal mammary gland tissue as well as in some breast cancer tissue (Sasano and Harada, 1998). Sanderson *et al.* (2000) reported the ability of ATR to induce aromatase mRNA in H295R human adrenocortical carcinoma cells. Estrogen acts on its receptors in the stroma to stimulate growth factors, including EGF, which are needed for ductal growth (Silberstein, 2001). Wiesen *et al.* (1999) found that EGFR is necessary to induce estrogen-dependent ductal growth and branching morphogenesis to stimulate epithelial growth and development.

To determine if ATR induces a change in aromatase or EGFR gene expression in vivo, RNA from the mammary gland was used for relative quantitation analysis (Ginzinger, 2002), and product size confirmed when run on an Agilent DNA chip against molecular weight markers (Figure 4.4). Threshold cycles for aromatase and EGFR were compared across exposure parameters (Table 4.4). Threshold detection of aromatase at PND33 was not different when averaged across the exposure parameter groups. However, when gene

expression was evaluated as fold induction, animals in the ATR-ATR group expressed significantly less aromatase when compared to C-C and ATR-C animals, meaning ATR-ATR offspring maintained less than half the gene copies present in controls ($p<0.05$). At PND40, neither threshold detection nor induction values differed significantly among the groups, even though fold induction of aromatase was increased in the ATR *in utero* exposure group (ATR-C) only.

Threshold detection for EGFR on PND33 in glands of ATR-ATR females was significantly different when compared to C-C and ATR-C levels, $p<0.02$. Suckling from an ATR exposed dam decreased fold induction values by nearly 3-5 fold compared to C-C ($p<0.01$ vs. C-ATR and $p<0.02$ vs. ATR-ATR) showing that there were far fewer copies of these genes present in the glands of ATR exposed offspring (1/3 to 1/5 the number of copies). By PND40 there were no statistical differences in threshold detection or fold induction values, but animals exposed to ATR *in utero* (ATR-C and ATR-ATR) had fold induction values of EGF-R two to nearly four times that of C-C animal.

Serum Hormone Measurements- To determine if circulating hormone levels played a role in delayed mammary gland development, serum was separated from trunk blood of animals at PND33, 40, and 58. Radioimmunoassays were performed to determine serum LH, PRL, and TSH concentrations. No correlation could be made with serum hormone status and mammary gland development delays. At PND33, the only change noted was a slight rise in the mean serum LH concentration (ng/ml) in ATR-C animals (0.78 ± 0.00), when compared to C-C and ATR-ATR animals (0.44 ± 0.06 and 0.47 ± 0.06, $p<0.01$; mean serum LH for C-ATR animals was 0.60 ± 0.08.), and although statistically significant, these LH values were within normal physiological range. There were no exposure-induced differences in hormone

concentrations at PND40. At PND58, TSH levels were decreased slightly in offspring of the ATR-ATR group ($p<0.02$ vs. C-C and ATR-C; Table 4.5). The values in all exposure groups were within expected range.

Reproductive Tissue Weights- Animals in diestrus were sacrificed on PND58. Anterior pituitary glands and reproductive organs were removed and weighed. All ATR exposure parameter groups had slightly increased pituitary weights, but only pituitary glands of animals in the ATR-ATR group were significantly increased ($p<0.05$ vs. C-C) when controlling for BW effects. As seen in Table 6, there were no statistical differences between the groups in reproductive organ weights. Even though the mean uterine weight (wet) of the ATR-ATR group was decreased nearly 10% from that of C-C group, it was not found to be statistically significant.

Discussion

The results of the studies presented here demonstrate that a brief exposure to 100mg/kg atrazine during the fetal period does delay mammary gland development in female offspring. The mammary glands of offspring either exposed *in utero* and/or that had suckled from an exposed dam took longer to develop into mature glands than those of controls. This was observed as early as PND4 and as late as PND40 when the control animals' glands were filled and grown to a relatively static resting state.

The studies suggest that there may be a critical period of fetal mammary gland development between GD15 and GD19 in which the developing gland may be sensitive to the effects of ATR, and that continued exposure of the developing offspring to ATR is not required for exposure-induced delays in mammary gland development seen in LE rats. Specifically, mammary gland development was delayed by in utero exposure to ATR, without nursing an exposed dam, and those effects were detectable as early as PND4. Low development scores were evident from PND 4-40 in the ATR-C group compared to controls. The results also suggest that ATR, either as a lactational exposure (ATR remaining after dosing exposure + breakdown products) or change in the composition of the milk received by the offspring, can negatively affect mammary gland development, as seen by the scores of the C-ATR group. However, the gestational exposure combined with milk consumption from an ATR-exposed dam had the greatest impact on mammary gland development. These data suggest that these effects are either length of exposure-dependent (assumes further exposure to ATR or its metabolites during lactation) or that ATR may be able to indirectly alter growth by impairing production of local or milk-mediated growth factors/hormones known to control mammary gland growth and development.

Previous studies published on the effects of ATR evaluated pubertal endpoints and/or reproductive tissues in animals that had been exposed directly to ATR for at least 20 days. They found that ATR delayed puberty and decreased uterine weights (Laws *et al.*, 2000, Ashby *et al.*, 2002). Ashby *et al.* (2002) and Eldridge *et al.* (1994) found that ATR exposure caused body, reproductive, and adrenal weights to be decreased. Irregular cycles and increased cycle length were also reported due to ATR exposure (Eldridge *et al.*, 1994, Cooper *et al.*, 1996, Eldridge *et al.*, 1999). In these studies, dams were exposed for 5 days during the latter part of the gestation period. There was a delay in VO seen in female offspring that suckled a treated dam coupled with higher BW of these animals. This is similar to what was reported for Wistar and SD rats (Laws *et al.*, 2000, Ashby *et al.*, 2002). However, a similar delay in VO was triggered in the present study by merely suckling an ATR-exposed dam. Limited differences in body or reproductive tissue weights were seen in the offspring exposed to ATR (10% decrease in wet uterine weight in ATR-ATR group compared to controls), which does not negate the possibility for effect in these tissues. Further, it does not appear that the short burst of atrazine during gestation alters development of normal estrous cyclicity patterns, or the number of animals demonstrating periods of constant diestrus or estrus, following VO.

It has been hypothesized that BW can play a role in the development of the mammary gland of rodents. However, only on PND4 were body weight differences detected (which were within normal range; reduced < 6%) and these were only found in the group born to and consuming milk from treated dams. At no time point was weight considered a significant covariate in our analyses, and offspring from the other exposure parameters had less

developed glands at times when no difference in body weight was detected from the control offspring.

Hormones are important to mammary gland development and changes in them could lead to altered mammary gland development. Although serum LH levels were elevated in one group of ATR-exposed animals, and TSH was decreased in yet another (ATR-ATR), these changes, which were not deemed biologically significant, did not persist over time. It appears that none of the endocrine endpoints evaluated contributed in a causal fashion to delayed mammary gland development.

The developmental delays observed in mammary glands of ATR-exposed pups suggest that there may be critical windows of fetal and neonatal mammary gland development that are sensitive to ATR exposure. We do not propose that ATR, per se, is directly responsible for altered growth of the pup gland early in life, but suggest that ATR causes changes in milk composition. Body weight, endocrine hormone levels, and other pubertal endpoints were not particularly useful in predicting mammary gland development in LE rats exposed prenatally to ATR.

Table 4.1 Effect of 100 mg/kg Atrazine on Estrous Cyclicity

			# Normal Cycles			
	N	IR	2	3	4	5
C-C*	31	7	4	9	5	6
ATR-C	38	9	6	11	8	4
C-ATR	25	6	9	3	5	2
ATR-ATR	32	1	12	10	6	3

***Dam-Milk Source, N= number of offspring, IR= irregular Cyclity, # Normal Cycles= Number of animals displaying consecutive normal cycles.**

Table 4.2. Effect of 100 mg/kg ATR on Offspring Mammary Gland Development				
Day	**C-C***	**ATR-C**	**C-ATR**	**ATR-ATR**
PND4	**3.5 ± 0.02**	**2.7 ± 0.2[a]**	**1.8 ± 0.2[a]**	**1.9 ± 0.3[a]**
PND22	**3.2 ± 0.3**	**2.1 ± 0.3[a]**	**1.9 ± 0.4[b]**	**1.8 ± 0.5[b]**
PND33	**2.9 ± 0.2**	**3.0 ± 0.0**	**2.2 ± 0.2**	**1.6 ± 0.5[c]**
PND40	**3.1 ± 0.2**	**2.4 ± 0.2**	**2.5 ± 0.2**	**2.0 ± 0.2[c]**

*** Dam Milk Source. Data presented as dame mean ± SE. Scoring: 1=stunted growth pattern; 4= normal growth for age. Dam N=2-5, with ≥ 5 offspring per exposure group at each time. Significant treatment effect by ANOVA; [a] p<0.02 vs. C-C, [b] p<0.05 vs. C-C, [c] p<0.01 vs. C-C.**

Table 4.3. Effect of 100 mg/kg ATR on BrdU Indices		
PND33	**% Structures Labeled**	**Labeling Score**
C-C*	**26.0 ± 6.04**	**1.7 ± 0.35**
ATR-C	**48.6 ± 10.7**	**2.8 ± 0.34**
C-ATR	**36.4 ± 4.67**	**1.7 ± 0.49**
ATR-ATR	**45.3 ± 15.1**	**1.8 ± 0.51**
PND40		
C-C*	**54.9 ± 34.5**	**1.2 ± 0.16**
ATR-C	**25.7 ± 10.5**	**0.9 ± 0.40**
C-ATR	**47.6 ± 4.83**	**2.1 ± 0.12a**
ATR-ATR	**53.2 ± 4.83**	**2.1 ± 0.14a**

***Dam-Milk Source. Data presented as dam mean ± SE. Labeling Score= subjective score for amount of labeling within structures; 1= lowest, 4= highest. Dam N=2-5, with ≥ 5 offspring per exposure group at each timepoint. Significant treatment effect by ANOVA; a p<0.01 vs. C-C.**

Table 4.4. Effect of 100 mg/kg ATR on Aromatase and EGF-R Gene Expression

	PND33		PND40	
Aromatase	**Threshold**	**Induction**	**Threshold**	**Induction**
C-C*	**34.2 ± 0.4**	**1.00 ± 0.0**	**35.2 ± 0.5**	**1.00 ± 0.00**
ATR-C	**34.2 ± 0.3**	**0.40 ± 0.8**	**33.7 ± 0.8**	**4.28 ± 3.0**
C-ATR	**34.5 ± 0.3**	**-0.41 ± 0.8**	**34.5 ± 0.5**	**1.30 ± 1.0**
ATR-ATR	**35.2 ± 0.4**	**-2.19 ± 0.5[a]**	**34.7 ± 0.2**	**1.10 ± 0.5**
EGF-R				
C-C*	**27.4 ± 0.4**	**1.00 ± 0.0**	**29.6 ± 0.7**	**1.00 ± 0.0**
ATR-C	**27.7 ± 0.2**	**-1.29 ± 0.2**	**27.9 ± 0.6**	**3.80 ± 1.0**
C-ATR	**28,6 ± 0.4**	**-2.75 ± 0.8[b]**	**29.2 ± 1.0**	**0.77 ± 2.0**
ATR-ATR	**29.4 ± 0.6[c]**	**-5.25 ± 2.0[c]**	**28.5 ± 0.4**	**2.07 ± 0.8**

***Dam-Milk Source. EGF-R= epidermal growth factor receptor. Data presented as dam mean threshold cycle or fold induction value ± SE. Dam N=2-5, with ≥ 5 offspring per exposure group at each timepoint. Significant treatment effect by ANOVA; [a] p<0.05 vs. C-C, ATR-C, [b] p<0.01 vs. C-C, [c] p<0.02 vs. C-C, ATR-C.**

Table 4.5. Effect of 100 mg/kg ATR on Serum Hormone Concentrations (/ml) at PND58				
	C-C*	ATR-C	C-ATR	ATR-ATR
LH (ng)	0.57 ± 0.06	0.61 ± 0.1	0.58 ± 0.1	0.49 ± 0.06
PRL (ng)	7.58 ± 2.0	8.21 ± 3.0	10.4 ± 3.0	7.70 ± 2.0
TSH (ng)	2.62 ± 0.2	2.81 ± 0.4	2.41 ± 0.2	1.81 ± 0.2[a]

***Dam-Milk Source. LH= luteinizing hormone, PRL= prolactin, TSH= thyroid stimulating hormone. Animals sacrificed in diestrus on PND58. Data presented as dam mean ± SE. Dam N≥6, with ≥ 14 offspring per exposure group. Significant treatment effect by ANOVA; [a] p<0.02 vs. C-C, ATR-C.**

Table 4.6. Effect of 100 mg/kg ATR on Reproductive Organ Weights				
	C-C*	ATR-C	C-ATR	ATR-ATR
Body Wt (g)	252 ± 7.3	252 ± 6.2	259 ± 6.3	241 ± 5.8
Pituitary (mg)	9.55 ± 0.48	10.1 ± 0.45	10.7 ± 0.54	10.9 ± 0.33a
Ovaries (mg)	110 ± 3.8	112 ± 2.9	101 ± 5.6	106 ± 3.5
Uterus (mg)	284 ± 11	275 ± 17	264 ± 7.3	258 ± 8.9

***Dam-Milk Source. Animals sacrificed in diestrus on PND58. Data presented as dam mean ± SE. Dam N≥6, with ≥ 14 offspring per exposure group. Significant treatment effect by ANOVA; [a] p<0.05 vs. C-C, adjusted for body weight.**

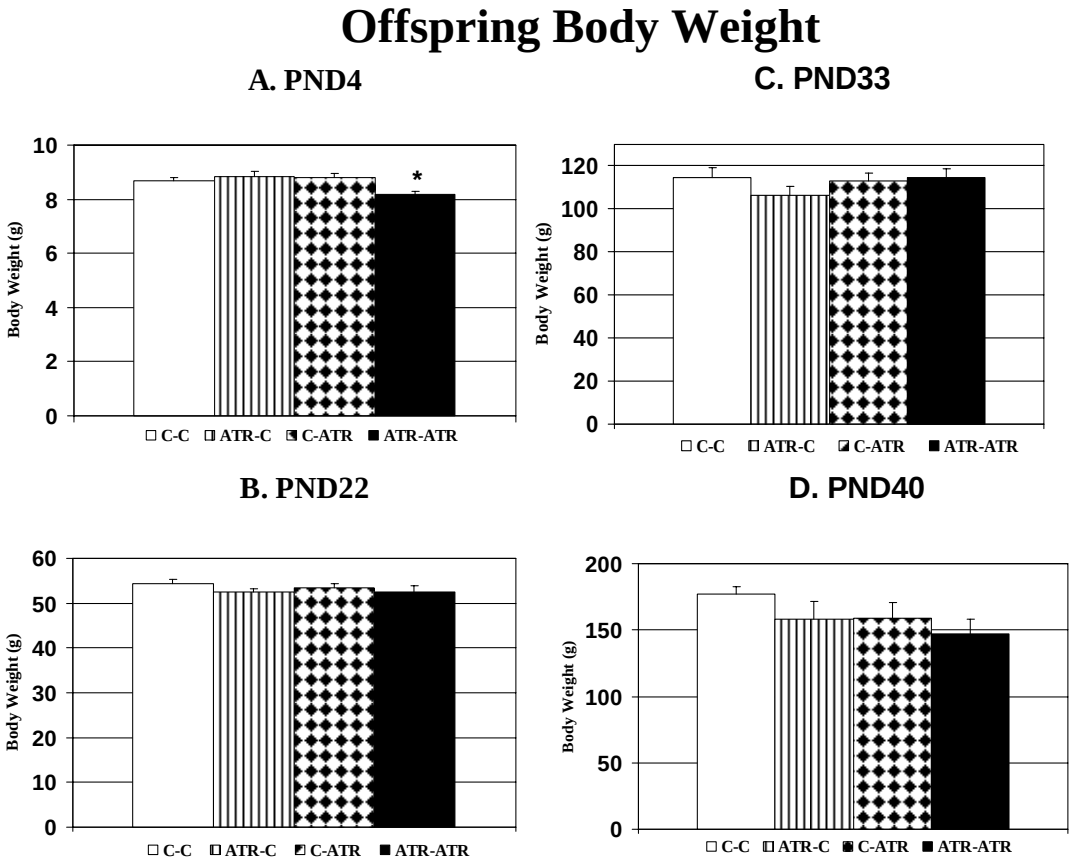


Figure 4.1. Body weight of pups exposed to ATR via exposure parameters and weighed on PND4, 22, 33, and 40. Data are presented as mean ± SE. Groups listed as dam-milk source. Pups in the ATR-ATR group were statistically smaller than those in the control group on PND4. * Significant treatment effect by ANOVA, $p<0.01$ vs. C-C (5.7%), ATR-C (7.9%), and C-ATR (6.8%). Weights at other timepoints were not different.

Age and Weight at Vaginal Opening

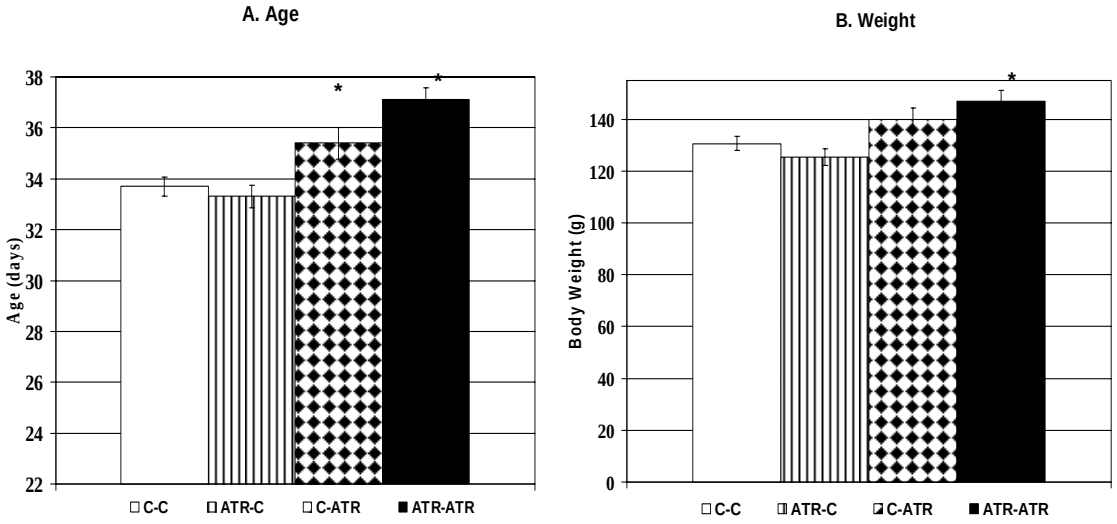


Figure 4.2. (A) Age (days) at the time of vaginal opening. Data are presented as mean ± SE. Groups listed as dam-milk source. *Significant treatment effect by ANOVA (LSM) and significantly different from C-C and ATR-C ($p<0.02$). (B) Mean ± SE of body weight (g) at the time of vaginal opening. Significant treatment effect by ANOVA (LSM); * $p<0.002$ vs. C-C and ATR-C.

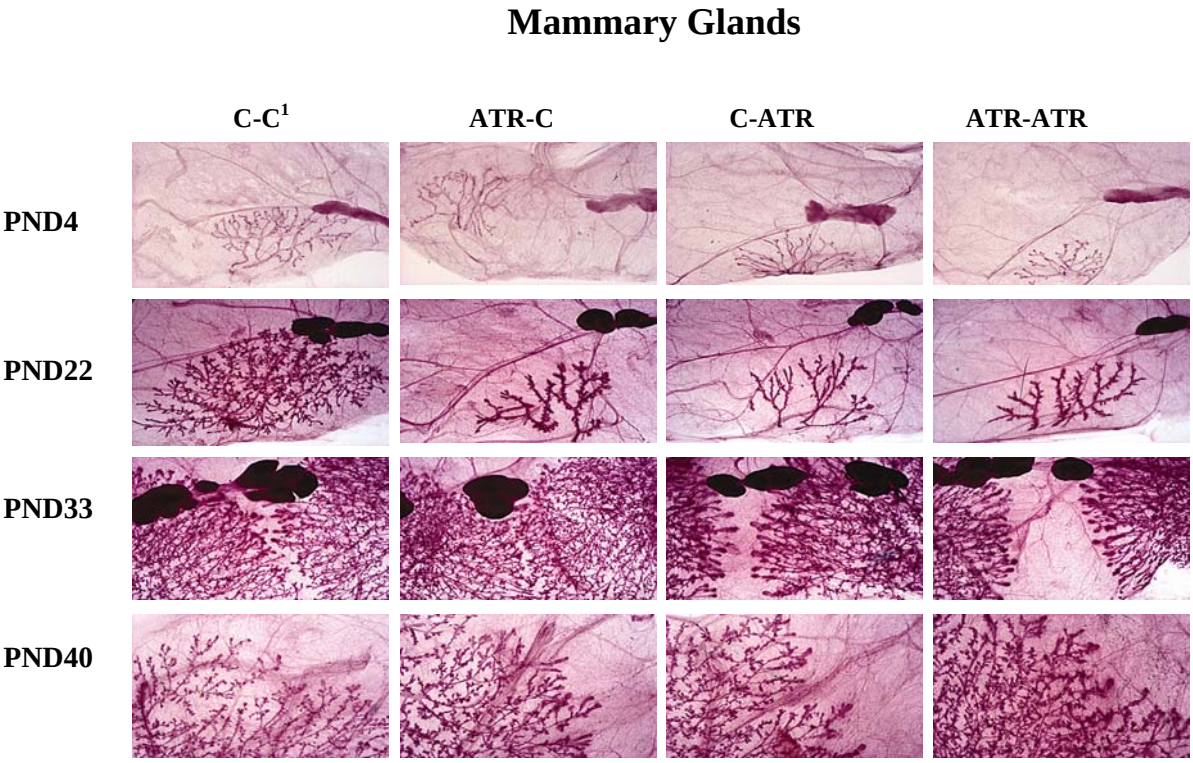


Figure 4.3. Mammary gland whole mount comparisons from female offspring following gestational exposure and cross-fostering. [1]Dam-milk source; C-Control, ATR-Atrazine. Altered mammary gland development was apparent in glands from animals within treatment exposure parameters. Pictures are representative of mean scores found in Table 3.

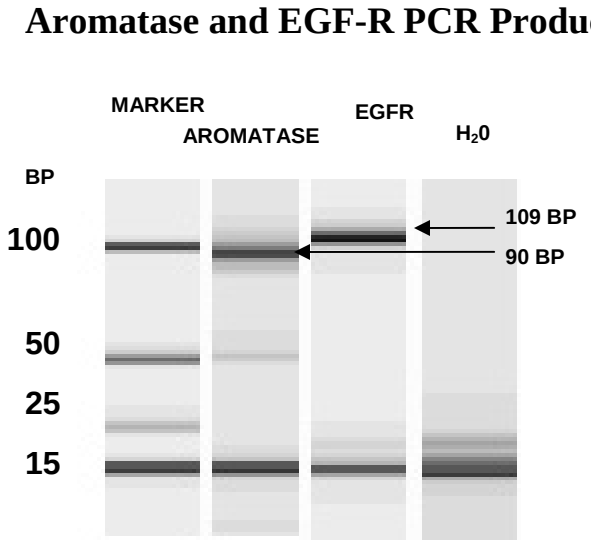


Figure 4.4. Products from real time(RT)-PCR. Lane 1 is molecular weight marker in base pairs (bp). Lane 2 is aromatase at expected size (90 bp). Land 3 is EGF-R at expected size (109 bp). Lane 4 is RT-PCR negative control (H_20 template).

CHAPTER 5

ATRAZINE AND CRITICAL PERIODS OF FETAL MAMMARY DEVELOPMENT

Summary

A 5-day exposure to 100 mg/kg/d ATR during GD15-19 in Long-Evans rats delayed mammary gland growth and development in female offspring. To determine if there is a gestational critical period in which the developing mammary tissue is most sensitive to the effects of ATR, the studies presented here evaluated the effect of ATR on mammary gland development as well as traditional pubertal indicators after 3 or 7-day exposures during the latter period of gestation. The 3-day exposure periods bracket the proposed times of rat mammary bud formation, bud size increase, mesenchyme differentiation, and epithelial cell proliferation into the fat pad. These studies also clarified that delayed offspring mammary growth and development following *in utero* ATR exposure is not caused by altered serum hormones or reduced mean fetal litter weights following what appears to be a period of maternal toxicity. These persistent mammary gland effects of prenatal atrazine exposure resulted in decreased weight gain in the second generation litters born to prenatally exposed females, demonstrating the effects on the F_1 mammary gland are adverse.

Methods

Animals-Time-pregnant Long Evans rats (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed in an AAALAC accredited facility, one per cage and given food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. They were maintained in a room with a 14:10 hour light cycle, 20-24°C and relative humidity of about 50%. Animal protocols were reviewed and approved by the National Health and Environmental Effects Research Laboratory (NHEERL), Institutional Animal Care and Use Committee. Animals were tested monthly for infectious diseases and remained negative for all NHEERL criteria.

Dosing Solution and Procedures- Critical Period Study - ATR (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. Timed-pregnant rats were treated in the morning and afternoon with 0 (vehicle) or 50 mg ATR/kg BW by oral gavage in 5 ml/kg dosing volume. This ATR reference dose (100 mg/kg/d) was chosen due to consistent reproductive endpoint effects observed in previous studies (Laws *et al.,* 2000; Rayner *et al.*, 2004; Stoker *et al.,* 1999).

Fetal Weight Study - ATR was prepared as a suspension in 1.0% methyl cellulose in distilled water. Timed- pregnant rats were treated with 0 (vehicle), 25 mg/kg, 50 mg/kg, or 100 mg ATR/kg BW by oral gavage in 5 ml/kg dosing volume.

Experimental Design- Critical Period Study-These studies were completed in two blocks. In each block, 40 pregnant LE dams were treated with vehicle (control) or 50 mg/kg ATR twice daily (N=8 dams/ exposure period/treatment, equal to 100 mg/kg/d). Control dams were dosed gestational days (GD) 13-19 and dams receiving ATR were dosed either on GD13-15,

GD15-17, GD17-19, or GD13-19. Litters were weighed and randomly equalized to 10 pups (6 females, 4 males) on PND4. At weaning, PND22 or 25, animals were weighed and separated into unisexual sibling groups, 2 female rats per cage. Females, at least 2/dam and 4 dams/group/block were sacrificed on PNDs 4 (from remaining pups following equalization of litters), 22, 25, 33, 46, and 67. The second block was extended to PND67 due to persistent effects seen in Block 1 mammary glands (ended on PND 46). Beginning on PND68, sibling females (N=4/group) of those sacrificed on PND67 and exhibiting normal estrous cycles were bred to control males. Second generation litters were weighed and randomly equalized to 10 pups on PND4. On PND11 of second generation, dams and pups were lactationally challenged (see below). Dams and pups were sacrificed after challenge. The data from an exposed dam's offspring were evaluated with the dam (pups/dam) as the experimental unit. No block effects were detected.

Fetal Weight Study- Forty pregnant LE dams were treated with vehicle (control), 25 mg/kg, 50 mg/kg or 100 mg/kg ATR daily (N=8 dams/ treatment) on GD15-19 as previously described (Rayner *et al.,* 2004). On GD20, 24 hours after the last dose was received, dams were sacrificed. Litters were removed from the dam and the sex and weight of each fetus were recorded. One control dam died before necropsy and one dam in the 50 mg/kg ATR group was not pregnant. The data from an exposed dam's offspring were evaluated with the dam (litter) as the experimental unit.

Vaginal Opening and Cyclicity-Beginning on PND29, female offspring (more than 24 females/group) were evaluated for vaginal opening (VO). The postnatal day of complete VO, and body weight on that day, were recorded. Daily vaginal smears were collected in Block 2 females beginning on PND37 and continued until PND 67 to determine the effect of

prenatal atrazine exposure on early cyclicity patterns following VO. Vaginal smears were read wet for the presence of leukocytes (metestrus/diestrus), nucleated epithelium (proestrus), or cornified epithelial cells (estrus) to determine cyclicity patterns on an American Optical low-power light microscope (100X) and day of cycle was recorded. Data were examined for 4-5 day normal cycles and the number of consecutive normal cycles was recorded for each animal. Episodes of persistent diestrus or estrus were recorded and the number of episodes per animal was compared across treatment. Irregular cyclicity was defined as those not presenting at least two consecutive normal cycles between PND37 and PND 67.

Necropsy- Necropsies were performed following an overnight and continued stay in a quiet holding area, and by using decapicones for animal transfer to reduce stress.

Critical Period Study-On PND33, 46, or 67, ≥2 females/dam were weighed and decapitated. On PND33 (N=6 dams/treatment group), mammary glands were removed for whole mounts from female offspring that had undergone VO in all dose groups, so that differences in mammary gland development could be evaluated separately from other facets of puberty. Trunk blood was collected and centrifuged for 30 minutes at 3000rpm (4°C) for serum. At PND46 (N>7 dams/group) mammary glands were removed for whole mounts. On PND67 (N>4 dams/group), trunk blood was collected and centrifuged for 30 minutes at 3000rpm (4°C) for serum from animals killed between 0800-1300 hours. The pituitary gland, ovaries, and uterus (wet) were removed and weighed. Mammary glands were removed for whole mounts. Normal cycling female siblings remaining after PND67 were retained for breeding experiments.

Fetal Weight Study-On GD20, dams (N>7/group) were decapitated, and trunk blood was collected and centrifuged as above for serum. The 4^{th} and 5^{th} mammary glands of each dam

were removed. Portions of these glands were processed for RNA, protein analyses, and histology. The uterus of each dam was removed and opened. The numbers of implantation sites and resorption sites were counted. Fetuses were removed from the membranes, rinsed in room temperature phosphate buffered saline, and blotted to remove excess liquid before weighing. After weight was recorded, the fetuses were decapitated and examined internally under a dissecting microscope to confirm their sex.

Mammary Whole Mounts and Histology- The 4th and 5th mammary glands were removed, fixed, and stained in carmine alum as a whole mount as previously described (Fenton *et al.*, 2002) on PNDs 4, 22, 25, 33, 46, and 67 (2-4 pups/dam). Mammary glands from second generation pups were removed and stained on PNDs 4 and 11. Flattened whole mounts were visualized and the epithelial outgrowth was measured to the closest millimeter (mm). Length measurements for area were taken from the nipple to the farthest point of branching. However, length measurements on PND46 were taken from the farthest branching of the 4th gland to the farthest branching of the 5th gland. Width measurements for area were taken from the two longest points of outgrowth. The whole mounts were subjectively scored (scale=1-4; 1=poor development/structure and 4=normal development/structure for each age group) within an age group, by two individual scorers some knowledge of treatment. Mammary gland histology sections were scored for ductile dilation, lipid accumulation, and developmental score. Scores ranged from 1 to 4 with 1 given to glands with ducts that are the least dilated, structures having the least lipid accumulation, and glands being the least developed and 4 given to glands having the most. Mammary glands representative of the mean score of the group were photographed on a Leica WILD M420 macroscope.

Radioimmunoassay- Sera were obtained from offspring that were decapitated on PNDs 33 and 67, and from dams at PND11 (second generation) for use in radioimmunoassays. Serum total testosterone, androstenedione, corticosterone, and progesterone were measured using Coat-a-Count Radioimmunoassay Kits obtained from Diagnostic Products Corporation (Los Angeles, CA). Serum estrone was measured using the DSL 8700 Estrone Radioimmunoassay kit and estradiol using the 3rd Generation Estradiol Radioimmunoassay kit from Diagnostic Systems Laboratories, Inc. (Webster, TX). Serum prolactin (PRL) was analyzed by radioimmunoassay using materials supplied by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases. All assays were run in duplicate. Stage of the estrous cycle was known at the time of necropsy and was used as a model variable in data analysis (see below).

Lactational Challenge- Dams and pups were moved to a quiet holding room on PND10. On PND11, dams were removed, weighed, and placed in clean individual cages with food and water *ad libitum*. Entire litters were weighed and pups individually weighed and allowed to remain in their own nest. Two hours later, dams were placed back with their pups. The amount of time it took for dams to nest on their young was recorded and dams were allowed to suckle their pups for 20 minutes after which dams were removed and decapitated. Trunk blood was taken for serum collection. The pituitary gland was removed, weighed, and discarded. Portions of the mammary gland were removed and placed in 10% buffered formalin. Litters were reweighed immediately, and mammary glands removed from female pups for whole mount analysis. The uterus of dams was removed and implantation sites counted. Male pups were euthanized.

Statistical Analysis- Dam means (pups/dam; litter as unit) were calculated for body and tissue weights, mammary gland scores, VO day, and serum hormones. Means and adjusted means relative to body weight were calculated for organ weights. Body weights, mammary gland scores, and serum hormone concentrations were evaluated for treatment effects within each age group by one-way analysis of variance (ANOVA, Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC). Organ weights and hormone concentrations were analyzed with respect to day of cycle by Mixed Model ANOVA and interactions between day of cycle and group were evaluated. Analysis of covariance, with body weight as a covariate, was used to evaluate the effects of treatment on mammary gland scores. Estrous cyclicity data were compared by Mantel-Haenszel analysis (Non-zero correlation and Row Mean Score Test). Resorption sites and pup death were analyzed in a contingency table using Fisher's exact test in Graphpad Instat (Graphpad Software, San Diego, CA). Block effects were evaluated but none were significant. Significant treatment effects were demonstrated by $p<0.05$ and specific p-values are indicated throughout this manuscript.

Results

Effects of Treatment on Dam and Fetal Weights:

Critical Period Study-These studies were initiated to directly evaluate effects of prenatal ATR exposure on mammary gland development, because our previous work (Rayner et al., 2004) demonstrated that a five-day ATR exposure (100 mg/kg BW/day) during late gestation could delay mammary gland development, as well as puberty.

Dam weight was recorded throughout the dosing periods and weight gain compared among groups (Table 5.1). During the GD13-15 exposure period, control dams (vehicle) gained an average of 20.4$\pm$3.4g. Dams exposed to 100 mg/kg BW/day ATR during GD13-15 and the first three days of GD13-19 gained no weight, significantly less than control dams, $p<0.0001$. Dams dosed with ATR GD15-17 gained significantly less weight than control dams, with weight gain reduced 82%, ($p<0.0001$), and ATR GD13-19 dams had a 35% reduced weight gain, ($p<0.0007$) on the middle three days of that exposure. In the final dosing period, dams in the ATR GD17-19 group had a 90% reduction in weight gain, ($p<0.0001$) compared to control dams. However, those exposed to ATR GD13-19 exhibited only a 26% reduction in weight gain during the GD 17-19 period, ($p<0.0403$). During the entire dosing period GD13-19, control dams gained a total of 78.2$\pm$6.1g and ATR-exposed dams less than half of that amount ($p<0.0001$), with most of that weight gained in the latter stages of the 7 day dose period. The dams treated with 100 mg/kg ATR gained significantly less weight than controls at all periods tested.

The combined mortality rates from PND4 (after equalizing litters) to weaning for both blocks were 24.6% for control, 33.2% for GD13-15, 40.5% for GD15-17, 30.0% for GD17-19, and 26.3% for GD13-19. Because the dam is the unit of measurement, the larger

than expected pup loss necessitated running an additional block of these studies. The cause of pup loss was unknown, but thoroughly investigated. It was not due to detectable illness, disease, or environmental stress. Further, the weights of these pups were similar to those in our previous studies and block effects were evaluated in all statistical analyses.

Fetal Weight Study- *In utero* exposure during gestation days (GD) 15-19 to100 mg ATR/kg maternal BW lead to lower pup weight just after birth (Rayner *et al.*, 2004) when pups also nursed from ATR-exposed dams. ATR-treated dams in this study exhibited significantly reduced weight gain during the exposure period when compared to vehicle-treated dams. A pronounced lack of weight gain during the dosing period in the Critical Period study described, stimulated a further study to determine if this reduction in maternal weight gain (maternal toxicity) played a role in term fetus number or mean fetal body weight.

Dam weight was recorded daily throughout the dosing periods and weight gain compared among groups. During the exposure period GD15-19, control dams gained an average of 51.2$\pm$3.0g (Table 5.2). Dams in the 25 mg/kg, 50mg/kg, and 100 mg/kg ATR, groups gained 6.1%, 42.0%, and 62.9%, less weight, respectively, than control dams during the exposure period. Dams in the 50 mg/kg and 100 mg/kg gained significantly less weight than controls ($p<0.002$), and the 25 mg/kg dose had no effect. Because the current mode of action of ATR includes altered serum prolactin, we evaluated the dam's circulating prolactin levels at GD20 (24 hours after the last dose). There were no statistically significant exposure-induced differences found in circulating prolactin levels among the groups (N>7 dams/group; 9.38$\pm$2.22 C; 6.40$\pm$0.78 ATR 25 mg/kg; 8.44$\pm$1.84 ATR 50 mg/kg; and 4.78$\pm$0.48 ATR 100 mg/kg), although the highest ATR dose reduced serum PRL to half that in controls.

Dam mammary glands were examined macroscopically to determine ductile dilation, lipid accumulation and developmental score at GD20. The glands from dams in the 50 mg/kg and 100 mg/kg groups had ducts that were dilated significantly more than those of control dams ($p<0.05$). Lipid accumulation was increased in all ATR exposed groups, $p<0.01$. Developmental scores were not found to be significantly different among the treatment groups.

The mean number of live fetuses per dam at necropsy did not differ among the treatment groups. The number of resorbed sites and dead fetuses were also evaluated (Table 5.2). Interestingly, dams treated with 100 mg/kg ATR had the greatest number of resorptions; a total of 13. That was a significant increase compared to controls which had no recognizable resorption sites, $p<0.001$ (Table 5.2). However, mean number of resorptions in controls of other species (0.82 resorptions/dam/study in Sprague Dawley rats; Charles River Laboratories, 1996) are typically higher than this (0) and therefore the increased loss in the ATR group may not be biologically relevant. Mean number of resorptions in the 100mg/kg group were also significantly greater than the means for the 25 mg/kg (2 sites total) and 50 mg/kg (3 sites total) groups, $p<0.01$ and $p<0.03$. The total number of dead fetuses at necropsy did not differ among the treatment groups.

The body weight of female fetuses was compared among groups (Figure 5.1) and no differences were found at GD20. When female and male weights were both evaluated together, no differences were found. These data taken together suggest that although ATR exposure during pregnancy may decrease maternal weight gain and increase the total number of reabsorbed fetuses, it had no effect on fetal weight gain, regardless of sex, over this short exposure period.

Offspring Endpoints – Critical Periods Study

Growth and Puberty- Body weight was compared among the female offspring of ATR treated dams at several time points. At PND4, there were no differences in the body weights of female offspring (10.2±0.2g C, 10.0±0.2g GD13-15, 10.2±0.2g GD15-17, 9.7±0.2g GD17-19, and 10.4±0.2g GD13-19). There were also no differences in body weight at PNDs 22, 25, 33, or 46. In fact, offspring from GD13-19 exposed dams weighed about the same as control offspring at PND4 (1.1% change vs. control), PND22 (1.7%), PND25 (1.3%), PND33 (7.0%) and PND46 (3.5%). However, at PND67, both GD15-17 and GD13-19 animals weighed significantly less than control, $p<0.01$.

Vaginal opening and estrous cyclicity were evaluated as physical signs of female reproductive development (Figure 5.2). Body weight at time of VO was not different among the treatment groups (panel A). VO occurred in control animals at PND32.6±0.33 (panel B). The offspring exposed to ATR on GD13-19 displayed a significant delay in VO (34.5±0.36, $p<0.0004$), similar to that seen previously (Rayner *et al.,* 2004). VO was not significantly delayed in any other dose group (33.5±0.41 GD13-15; 32.9+0.31 GD15-17; and 33.2±0.36 GD17-19).

Estrous cyclicity patterns of the female offspring were observed from PND37 to PND67. The number of consecutive normal cycles (4-5 days) were determined and analyzed according to treatment for all animals. The majority of animals in each group had three to four consecutive normal cycles, and less than two animals per group displayed persistent estrus. No significant differences due to treatment were found in the number of consecutive normal cycles or irregular cycles among the treatment groups.

Mammary Gland Development- Mammary glands were removed from female offspring in all groups on PNDs 4, 22, 25, 33, 46, and 67 and examined to determine if epithelial development of the mammary gland was affected by gestational ATR exposure. Stained epithelia were measured (area (mm^2) and length (mm)) to observe differences in outgrowth into the fat pad (Table 5.3). Mammary gland development in female offspring was scored through whole mount analysis (Table 5.4, Figure 5.3).

The area (mm^2) of the mammary gland on PND4 in all 3-day ATR exposed groups was significantly smaller, $p<0.001$, compared to control offspring (22.5 ± 2.7 mm^2), with glands from the GD13-19 exposure group measuring less than half the size of controls (10.9 ± 1.13 mm^2). As expected and repeating previously reported ATR-induced delays (Rayner et al., 2004), glands from offspring exposed GD15-19 (GD15-17, 17-19) and GD13-19 were smaller and developmentally delayed in branching and ductal structures compared to glands taken from control offspring (Figure 5.3, Table 5.4). There is no evidence that these mammary developmental deficits are due to overall growth inhibition of the pups (either during gestation or after birth) due to treatment.

Weaning occurred at either PND22 (Block 2) or 25 (Block 1), and mammary glands were taken from exposed offspring of equivalent BW. The area of the fourth gland remained significantly smaller in exposed offspring at PND22, $p<0.03$ (Table 5.3), with the exception of GD13-15 offspring, but the distance between the fourth and fifth glands were not different. The mammary glands of GD17-19 and GD13-19 offspring displayed fewer terminal end buds and lateral branches, and had not migrated as far through the fat pad as controls (Table 5.4, Figure 5.4, top). The area of the fourth gland remained significantly smaller in ATR-exposed

offspring at PND25 (Table 5.3), and the distance between the glands was greater than the same distance in the control females' glands, $p<0.04$. Control female offspring displayed normal mammary branching with distended terminal end buds and terminal ducts. Glands of females in ATR-exposed groups were not as well developed. Those glands had poor migration of epithelium into the fat pad, and were sparse in appearance with fewer lateral branches than control (Table 5.4, Figure 5.3).

By the peri-pubertal time point, PND 33, none of the ATR-exposed offspring glands were statistically significantly smaller in area than controls (Table 5.3). Glands from control females displayed terminal end buds only on the most distal ends, lobules were present, and the fourth and fifth glands had grown close together (Figure 5.3). Even though glands from exposed offspring were not smaller in area, the glands received lower developmental scores than controls. ATR-exposed gland displayed abundant terminal end buds on 2-3 sides of the epithelial tree, limited lobules, and sparse branching (Table 5.4). Figure 3 shows the distance between the fourth and fifth glands at PND33 in addition to terminal end buds.

At early adulthood, PND46, glands of ATR-exposed offspring were similar in length to controls (Table 5.3). The 4^{th} and 5^{th} glands had grown together by that age and it was difficult to measure the area of each individual gland. However, whole mount analysis of the mammary glands showed that developmentally, ATR-exposed glands were significantly different from controls. Glands of control animals had very few terminal end buds left in the gland and the mammary gland was very dense with epithelial branching (Figure 5.4, bottom). Most glands of control animals resided in a resting state normally found in adult female rats. The development of mature gland structures was delayed in ATR-exposed offspring. Glands from exposed offspring were less dense and still retained many terminal end buds with most

seen in GD17-19 and GD13-19 (arrows in Figure 5.4), leading to consistently low developmental scores (Table 5.4). At a later point in adulthood when all females should have been sexually mature (PND67), ATR-exposed glands were still developmentally delayed and contained many large lobular units, with only moderate epithelial branching. Glands removed from female offspring in the GD17-19 and 13-19 groups received epithelial development scores that were statistically equivalent over time (PND4-67; Table 5.4). Control glands had few complex lobules and dense branching throughout the gland and remained in the resting state described above (not shown).

Serum Hormone Measurements-To determine if ATR exposure affected circulating hormone levels, serum was separated from trunk blood of animals at PND33 and 67. At PND33, corticosterone concentrations were significantly increased in the GD15-17 group, $p<0.009$; 72.1 ± 26.2, C vs. 193.9 ± 21.8, GD15-17. Although no stress events were noted, we realized this large increase could be due to an unknown environmental stressor prior to/during necropsy. Total testosterone in GD13-19 animals was doubled compared to controls, $p<0.04$; 0.203 ± 0.06, C vs. 0.419 ± 0.08, GD13-19. No additional differences in hormone concentrations evaluated at PND33 were found.

At PND67 (Table 5.5), there were no consistent exposure-induced differences in hormone concentrations with respect to day of cycle, but there were some interesting trends observed when all animals were compared to control means. Estrone and its precursor androstenedione were slightly decreased in GD17-19 and GD13-19 offspring. Estradiol was slightly increased in GD15-17, GD17-19, and GD13-19 offspring, whereas its precursor testosterone was nearly doubled in GD13-19 ATR exposed animals (similar to that seen in PND 33 animals of the same dose group). Steroid hormone ratios, particularly those

controlled by aromatase and 17-β hydroxysteroid dehydrogenase, were evaluated but no statistical differences among the groups were detected.

Reproductive Tissue Weights-Females from each group were sacrificed on PND67 (one day before siblings were bred). In addition to the previous data described, the ovaries and uterus were removed and weighed. As seen in Table 5.5, there were no statistical differences among the groups in these organ weights with respect to day of cycle, even though there was a 14.3% to 38.9% increase in total ovary weight in the ATR-exposed animals versus controls. There was also a 19.4% to 57.7% increase in wet uterine weight compared to controls in the ATR-exposed females.

Consequences of brief prenatal ATR exposure on 2nd Generation

The offspring of control and ATR-treated dams were bred to control LE males (beginning PND68) to determine if the females exposed gestationally to ATR, and exhibiting delayed mammary gland development, would have difficulty sustaining their young. Of the 20 normal cycling females chosen to breed (4/group), only one female in the GD17-19 group did not conceive. One female in the GD13-19 group conceived, delivered 2 pups, but dam and pups died before PND4. On PND4 post partum, individual pups in each litter (N>3) were weighed and the sex of each was determined. Litters were equalized to 10 pups if possible. On PND11, dams were removed from their pups for two hours during a lactational challenge as described.

Female pups in the groups GD17-19 and GD13-19 were significantly smaller than those of control ($p<0.003$ and 0.02), with reduction in body weight of 14.8% and 12.5%, respectively. Male pups in these same groups were also smaller than control, $p<0.002$ and 0.0001, and had body weight reduction of 15.6% and 16.7% (Figure 5.4 A). Mammary

glands were removed from the female pups at PND4 and scored for development and the area of the gland measured. Whole mount analysis of the fourth and fifth mammary gland at PND4 showed that mammary epithelial development of pups taken from GD17-19 and GD13-19 dams had few ductal buds from lateral branches and were undersized compared to glands taken from other groups. Control, GD13-15, and GD15-17 offspring glands displayed small buds on primary branches and moderate branching within the gland, and received similar developmental scores. When mammary gland scores were compared using body weight as a cofactor, glands from GD17-19 and 13-19 were no longer considered significantly different. The areas of the 4th mammary gland were not different among the groups. It should be mentioned that the mean body weight of pups on PND 4 in the GD13-15 and GD15-17 ATR exposure group (male and female) were significantly greater than the control group. The development of mammary glands in female siblings from both ATR exposure groups was known to be similar to controls when they were mated.

There were no differences in dam body weight across group (Table 5.6). The anterior pituitary glands (source of prolactin and decreased weight from ATR exposure, Laws *et al.*, 2000) were removed and weights were found to be similar across dose group. Although highly variable, dam nesting time was not different and litter weight gain during the nursing period was not different among the treatment groups (not shown). Estradiol, progesterone, and prolactin concentrations were measured from serum taken from the trunk blood of dams at PND11. Lower concentrations of estradiol were found in GD13-15 and GD13-19 dams, $p<0.0006$ and 0.02, respectively. No other differences were found in hormone concentration among the groups, even though some values were quite varied (e.g. PRL GD17-19).

Body weights of the pups were recorded at PND11, and again GD17-19 and GD13-19 had dramatically reduced weight in both sex groups; female $p<0.0001$ (25%) and 0.0003 (23.8%), male $p<0.0001$ (23.9%) and 0.002 (13%) (Figure 5.4 B). Mammary glands removed from females at PND11 were scored and measured. Glands taken from GD13-15, GD17-19, and GD13-19 scored significantly lower than control and GD15-17 glands. The areas of glands from GD17-19 and GD13-19 were reduced. As noted above, the pups from GD17-19 and GD13-19 dams were smaller at PND11. This led us to evaluate mammary gland scores and area using body weight as the covariate. Mammary gland development was not found to be significantly different on this basis, demonstrating that body weight may have been causal in the delayed development observed at this time point, unlike the delays in F_1 mammary development where body weight was not a significant factor in mammary gland size or developmental score. Delays in mammary gland development were not evaluated past PND11 in the F_2, but have the potential to exacerbate the effects of a brief prenatal ATR exposure in two generations of offspring to a third.

Discussion

The results of the studies presented here demonstrate that exposures as brief as three days to 100 mg/kg atrazine during late gestation caused delayed mammary gland development in the female offspring. These results also suggest that the fetal mammary gland may be most sensitive to the effects of ATR during the latter part of pregnancy, during the time of epithelial outgrowth. The mammary glands of offspring exposed GD17-19 took longer to develop into mature glands. This developmental deficit was observed as early as PND4 and as late as PND67, when the animals were sexually mature.

These studies suggest that GD17-19 may be the most important period of fetal mammary gland development, showing sensitivity to ATR that was consistent with the 7-day exposure. The previous chapter showed that a five-day *in utero* exposure to ATR during GD15-19 delayed mammary gland development in the female offspring. It was reported that gestational exposure combined with milk consumption from an ATR-treated dam (ATR-ATR) led to more severely delayed mammary glands than those exposed only *in utero* or lactationally. In the present study, a three-day *in utero* exposure, especially during GD17-19, caused mammary gland developmental delays similar to that found in the five-day ATR-ATR exposed, and the GD13-19 offspring (7-day exposed; this study), framing the narrowest sensitive period of mammary gland development to non-lipophilic environmental agents to date.

It has been suggested that maternal/fetal weight gain and/or early pup weight gain might play a role in offspring reproductive tissue development, and in this case, specifically the mammary gland. The previous chapter and data presented here demonstrate that F1 offspring body weight at GD20, PND4 or after was not a significant variable influencing

mammary gland developmental delays induced by ATR. The present fetal weight study showed that dams treated with 50 mg/kg or 100mg/kg of ATR during GD15-19 gained significantly less weight than control dams, but was without effect on litter sizes or fetal/pup weight. During the critical period study, dams treated with 100 mg/kg BW/day ATR during the three-day exposure period gained significantly less weight than control dams during the same time period, in fact, during early treatment periods they gained no weight. Dams treated during GD13-19 were reduced in weight gain by almost half of control dams, but there were no differences in female offspring weight in any of the groups following birth. However, differences in F_1 (specifically) mammary gland development were apparent in groups exposed to ATR, even after three days. Further, as noted in Table 5.6, there was no significant weight difference in F_1 dams used in the breeding study. Importantly, however, those same animals with delayed mammary gland development at breeding (to generate the F2 offspring) raised pups that were significantly (from 12-25%) smaller than controls, suggesting that the GD17-19 and 13-19 ATR-exposed dams were not able to produce quality and/or quantity of milk necessary to sustain the body weight of their offspring.

In an unpublished study prepared by Ciba-Geigy and accepted by the EPA (Mainiero *et al.*, 1987), rats were exposed to ATR over 2-generations. Male and female rats were treated through their diet with approximately 0.5, 5, and 50 mg/kg/day ATR prior to mating and then females were treated during gestation and lactation. The first generation of pups were weaned and treated through the diet for 12 weeks prior to mating. The only changes noted in both sets of parents were weight reductions at the high dose, but no changes were noted in the offspring due to treatment in the reproductive parameters evaluated including fertility, postnatal mortality, or developmental delays. This study, over two generations,

consisted of treating only F0 pregnant dams 3 or 7 days with ATR by oral gavage. Female offspring displayed persistent delayed mammary gland development (an end point not evaluated in NTP-like 2-generational studies) but appeared to have no changes in fertility or litter size. It was noted that F2 litters in the GD17-19 and GD13-19 ATR exposure groups were significantly reduced in body weight at PND4 and PND11. The mammary glands of these F2 offspring were also smaller and less developed than the other groups and this was found to be due to reduced body weight. These data suggest that late gestational ATR exposure has an indirect adverse effect on body weight of the next generation. The differences in results of the two studies mentioned above may be due to inconsistencies in time of breeding or differences in dose. Delayed mammary gland development was observed in the siblings of the females bred on PND68. It is possible that F2 effects may not have been seen if the females had been bred at a later time, such as 90-120 days as is typically seen in 2-generational studies. There is no proof that this effect is permanent, just long-lasting.

In conclusion, mammary gland developmental delays observed in all groups of ATR-exposed pups suggest that GD17-19 is a sensitive window of fetal mammary gland development. While traditional endpoints were measured (body weight, puberty, and serum hormone concentrations), they were not associated with mammary gland development effects in LE rats.

Table 5.1. Critical Period Study: Effect of 100 mg/kg ATR on Maternal Weight Gain (g)

	N	GD13-15	GD15-17	GD17-19	GD13-19
Control	**15**	**20.4 ± 3.4**	**27.3 ±1.4**	**30.5 ± 2.2**	**78.2 ± 6.1**
ATR GD13-15	**16**	**0.18 ± 1.8a**			
ATR GD15-19	**16**		**4.85 ± 2.2[a]**		
ATR GD17-19	**16**			**3.02 ± 3.3[a]**	
ATR GD13-19	**16**	**-0.28 ± 1.5[a]**	**17.7 ± 1.7[a]**	**22.7 ± 2.0[b]**	**40.1 ± 3.4[a]**

N=number of dams dosed on gestations days (GD) as shown. Data shown as dam mean ± SE. Significant treatment effect by ANOVA, [a] p<0.01, [b] p<0.04

Table 5.2. Treatment Effects on Dam Weight Gain and Fetal Outcome on GD20.

	N	Maternal Weight Gain	Litter Means	Resorbed Fetus	Dead Fetus
Control	**7**	**51.2 ± 3.0**	**13.7 ± 1.0**	**0 ± 0.0**	**0 ±0.0**
25 mg/kg ATR	**8**	**48.1 ± 3.9**	**14.6 ± 0.5**	**0.25 ± 0.2**	**0.12 ± 0.12**
50 mg/kg ATR	**8**	**29.7 ± 6.7[a]**	**13.9 ± 0.6**	**0.43 ± 0.2**	**0 ± 0.0**
100 mg/kg ATR	**7**	**19.0 ± 3.2[a]**	**11.1 ± 1.0**	**1.62 ± 1.0[c]**	**0.24 ± 0.25**

N=number of dams. Weight gain during GD15-19 dosing period. *Dead but not resorbed fetus. Data presented as litter mean ± SE. Significant treatment effect by ANOVA, a p<0.01 vs. Control. Significant effect by Firsher's exact test; two sided p<0.001 vs. Control.

Table 5.3 Effect of 100 mg/kg ATR on 4th Mammary Gland Areas (mm2) PNDs4-33 and Epithelial Length (mm) at PND46.

Day	Control	GD13-15	GD15-17	GD17-19	GD13-19
PND4	22.5 ± 2.7	14.3 ± 1.2[a]	14.0 ± 1.3[a]	21.7 ± 0.84[a]	10.9 ± 1.1[a]
PND22	77.4 ± 9.0	69.2 ± 13	43.4 ± 2.9[a]	48.5 ± 10[b]	30.6 ± 6,8[a]
PND25	126 ± 12	85.4 ± 5.2[a]	75.5 ± 8.3[a]	67.6 ± 6.2[a]	57.7 ± 11[a]
PND33	193 ± 11	176 ± 9.8	196 ± 11	175 ± 9.1	152 ± 8.7
PND46	52.2 ± 0.62	49.2 ± 1.2	50.0 ± 0.80	52.4 ± 1.8	48.8 ± 1.3

PND=postnatal day. Dam ≥ 8, with ≥ 5 offspring per group at each timepoint. Data presented as dam mean ± SE. Significant treatment effect by ANOVA, [a] p<0.001 vs. Control, [b] p<0.03 vs. Control.

Table 5.4. Effect of 100 mg/kg ATR (Evaluated by Subjective Scoring) on Mammary Gland Development

Day	Control	GD13-15	GD15-17	GD17-19	GD13-19
PND4	3.2 ± 0.2	2.8 ± 0.2	2.3 ± 0.2[a]	1.9 ± 0.2[a]	2.1 ± 0.1[a]
PND22	3.2 ± 0.3	2.7 ± 0.4	2.4 ± 0.2	2.0 ± 0.1[a]	1.6 ± 0.2[a]
PND25	3.3 ± 0.2	2.7 ± 0.1[b]	2.4 ± 0.2[a]	2.2 ± 0.2[a]	2.2 ± 0.2[a]
PND33	3.5 ± 0.1	3.0 ± 0.1[a]	2.7 ± 0.1[a]	2.7 ± 0.1[a]	2.6 ± 0.1[a]
PND46	3.7 ± 0.1	3.0 ± 0.1[a]	3.0 ± 0.1[a]	2.9 ± 0.1[a]	2.7 ± 0.1[a]
PND67	3.4 ± 0.1	2.8 ± 0.2[b]	2.8 ± 0.2[b]	2.6 ± 0.2[a]	2.4 ± 0.1[a]

GD=gestation day, PND=postnatal day. Dam ≥ 8, with ≥ 5 offspring per group at each timepoint. Scoring: 1=stunted growth pattern; 4= normal growth for age. Data presented as dam mean ± SE. Significant treatment effect by ANOVA, [a] p<0.01 vs. Control, [b] p<0.05 vs. Control.

Table 5.5. Lack of Effect of 100 mg/kg ATR on Reproductive Organ Weights and Serum Hormone Concentrations (/ml) at PND67.

	Control	GD13-15	GD15-17	GD17-19	GD13-19
Body Wt (g)	273 ± 3.5	261 ± 10	235 ± 5.3[a]	257 ± 5.0	244 ± 5.7[a]
Ovaries (mg)	116 ± 8.4	149 ± 10	146 ± 15	161 ± 21	132 ± 4.7
Uterus (mg)	310 ± 31	370 ± 35	430 ± 57	489 ± 112	418 ± 36
Progesterone (ng)	8.80 ±2.7	7.60 ± 2.2	5.40 ± 2.5	11.6 ± 7.8	6.50 ± 1.6
Corticosterone (ng)	128 ± 17	119 ± 31	107 ± 11	113 ± 42	128 ± 22
Androstenedione (ng)	0.60 ± 0.19	0.64 ± 0.12	0.46 ± 0.19	0.39 ±0.08	0.35 ±0.08
Estrone (pg)	57/3 ± 9.1	58.3 ± 5.6	68.7 ±8.3	43.6 ± 14	54.8 ± 5.0
Testosterone (ng)	0.16 ± 0.04	0.19 ± 0.07	0.20 ± 0.10	0.15± 0.09	0.29 ± 0.05
Estradiol (pg)	51.7 ± 2.3	46.6 ± 3.9	70.7 ± 33	59.3 ± 15	56.3 ± 4.8
Prolactin (ng)	6.90 ± 2.9	3.60 ± 0.30	4.50 ± 2.1	9.40 ± 4.3	8.80 ± 4.9

GD=gestation day. Dam N ≥ 4 (>2 pups/dam). Data presented as dam mean ± SE. Significant treatment effect by ANOVA; [a] p<0.01 vs. Control.

Table 5.6. Effect of 100 mg/kg ATR on Lactational Challenge and Serum Hormone Concentrations (/ml) at PND11

	Control	GD13-15	GD15-17	GD17-19	GD13-19
Body Wt (g)	393 ± 19	386 ± 16	401 ±15	353 ± 16	391 ± 13
Pituitary (mg)	15.7 ± 0.7	12.5 ± 1.0	15.4 ±2.0	13.7 ± 0.4	16.9 ± 2.4
Nesting Time (sec)	287 ± 111	638 ± 121	390 ± 71	297 ± 68	307 ± 61
Estradiol (pg)	49.1 ± 2.9	32.0 ± 1.4[a]	43.9 ± 6.3	41.9 ± 2.9	33.6 ± 2.3[a]
Progesterone (ng)	58.8 ± 15	62.9 ± 4.8	44.7 ±10	42.9 ± 21	62.2 ± 2.4
Prolactin (ng)	141 ± 50	109 ±27	144 ± 68	21.0 ± 7.3	107 ± 57

GD=gestation day, PND=postnatal day. Data presented as dam mean ± SE. Significant treatment effect by ANOVA, [a] $p<0.02$ vs. Control.

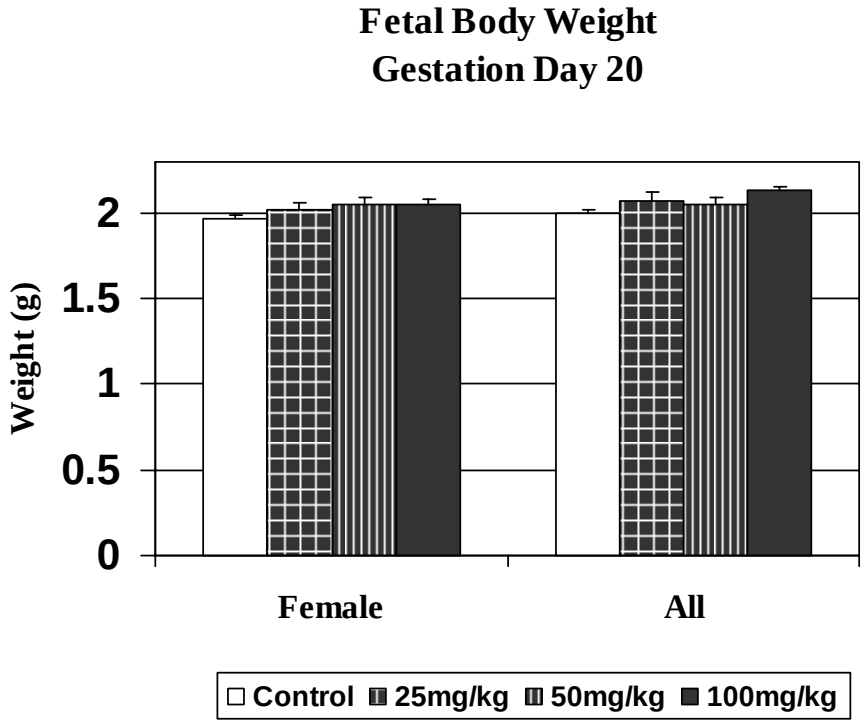


Figure 5.1. Litter mean (N>7/group) body weights (g) of GD20 pups exposed to increasing concentrations of atrazine during GD15-19 (25, 50, and 100 mg/kg). Data are presented as litter mean $\pm$ SE. Weights at this timepoint were not reduced compared to control in any dose group.

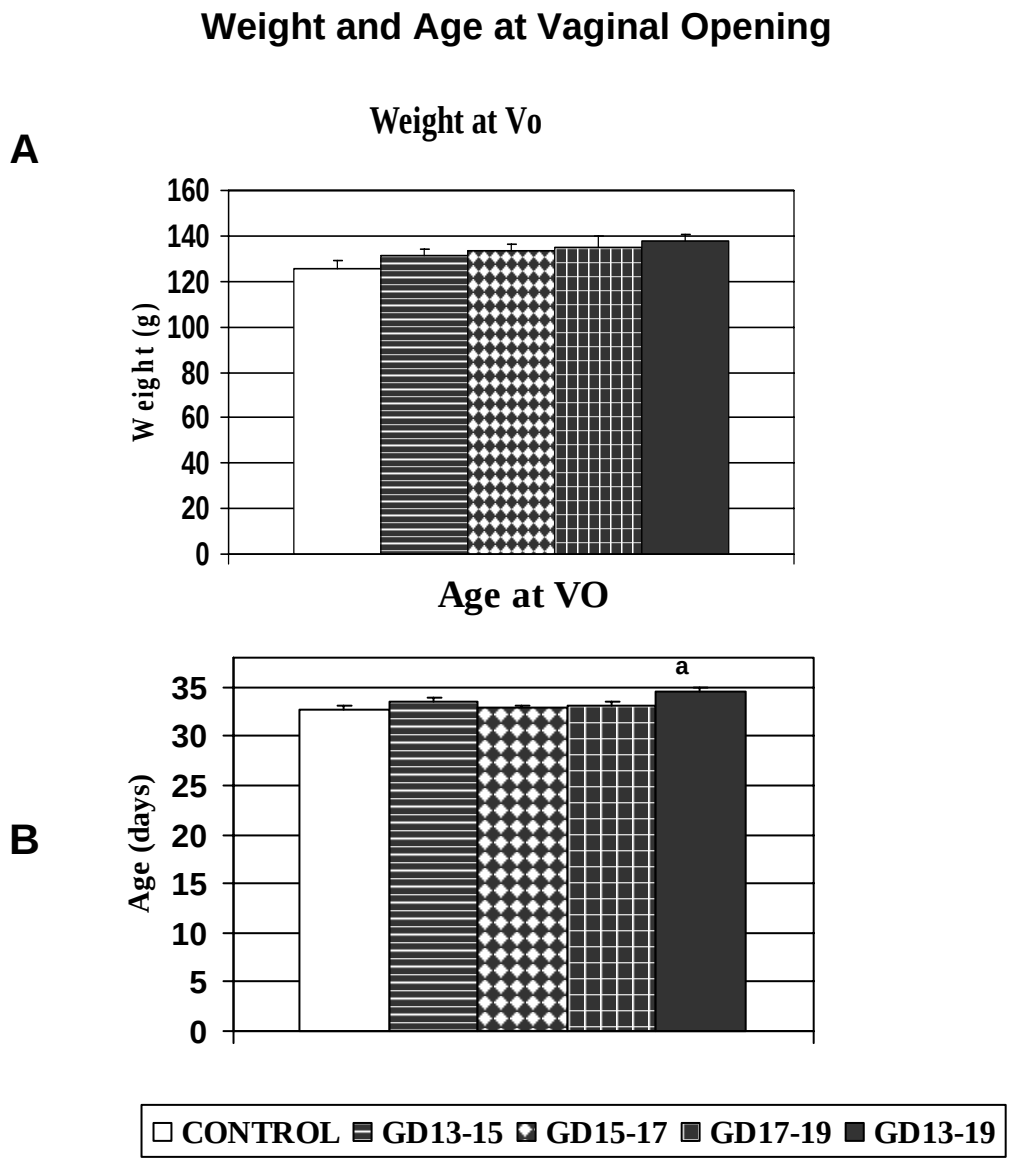


Figure 5.2. Effect of gestational exposure to 100 mg atrazine/kg BW/day on female pubertal body weights and age at time of vaginal opening (VO). (A) Litter mean (N>4 litters) body weight (g) at the time of vaginal opening. (B) Litter mean age (days) at the time of vaginal opening. Data are presented as mean $\pm$ SE. [a]Significant treatment effect by ANOVA (LSM) and significantly different from control ($p<0.0004$).

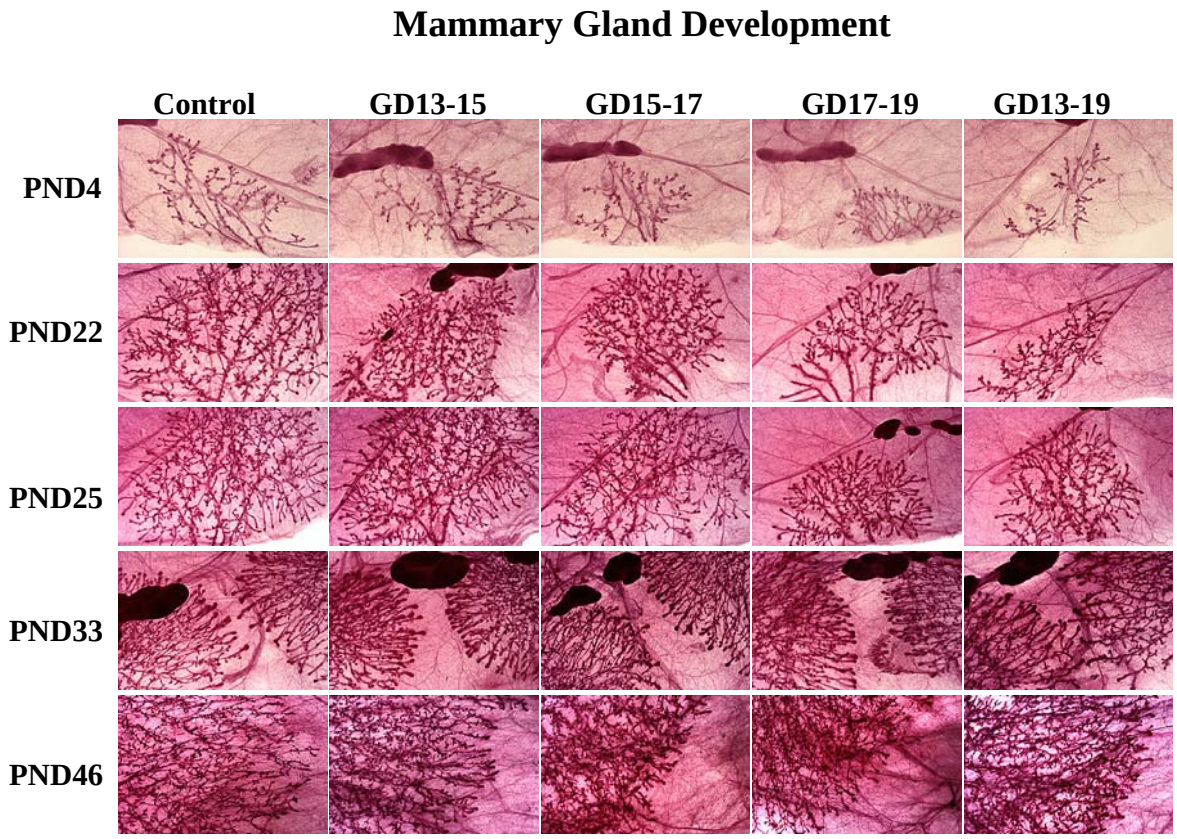


Figure 5.3. Critical window for effects of prenatal exposure to 100 mg atrazine/kg BW/day on mammary gland development. Mammary gland whole mounts were prepared from female offspring on postnatal days (PND) 4, 22, 25, 33, and 46 following ATR exposure on gestational days (GD) 13-15, 15-17, 17-19, or 13-19. Photos demonstrate the mean scores found in Table 5.4.

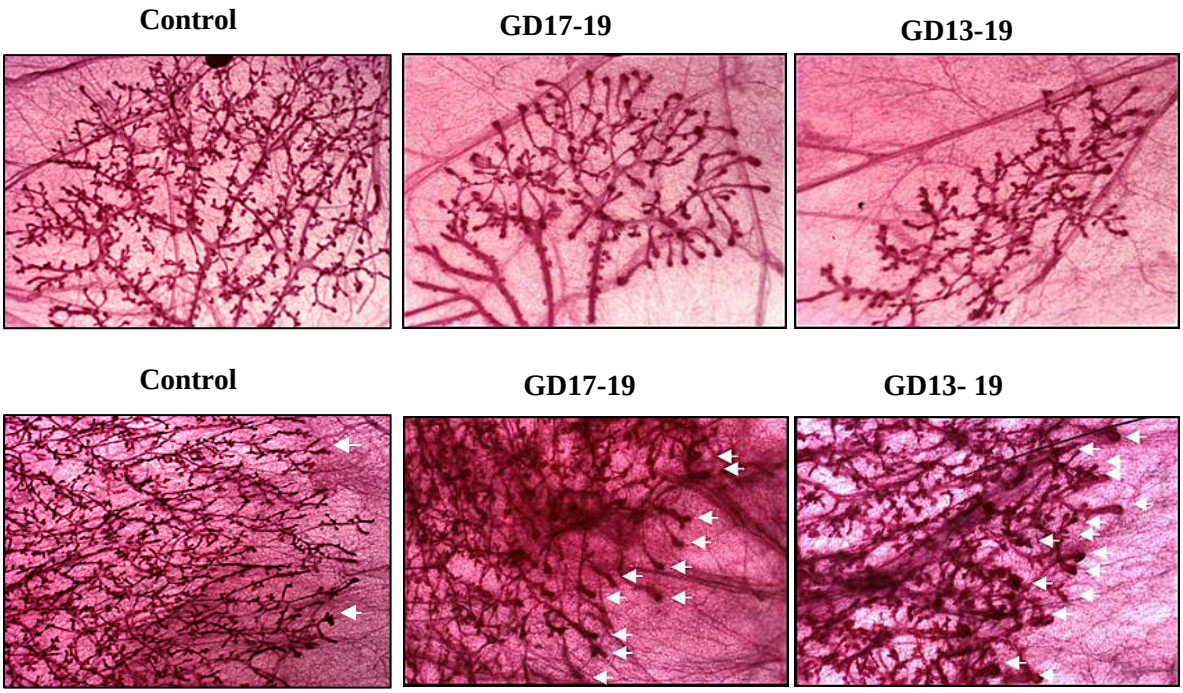


Figure 5.4. Enlarged picture of PND 22 mammary gland from control, GD17-19, and GD13-19, demonstrating fewer branches, delayed migration through the fat pad, and sparse appearance of atrazine exposed glands (top). Enlarged picture of PND 46 mammary gland from control, GD17-19, and GD13-19 (bottom). Glands taken from GD17-19 animals presented numerous terminal end buds (arrows) compared to few to none in control animals. The glands from the 3-day exposure period (GD17-19) closely resembled the glands from 7-day exposure (GD13-19).

Second Generation Offspring Weight

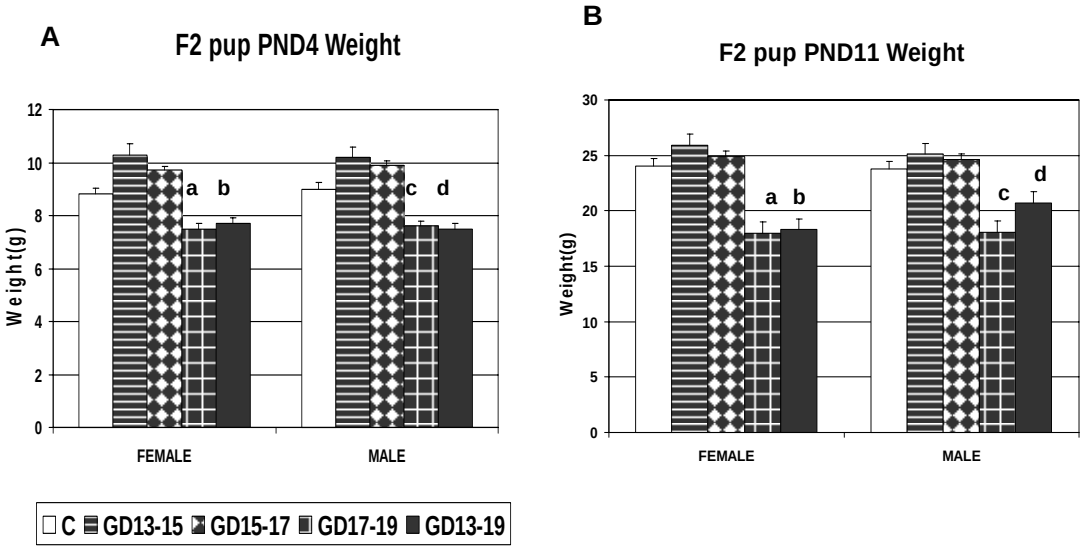


Figure 5.5. Decreased mean body weight (BW, g) following gestational exposure to 100 mg atrazine/kg BW/day of 2nd generation pups (F2) on postnatal days (PND) 4 and 11. Data are presented separately for male and female pups as litter mean $\pm$ SE. N>3 dams with >8 pups/dam (A) BW on PND4. Significant treatment effect by ANOVA (LSM) and reduction in BW; different from control a= $p<0.01$ (14.8%); b= $p<0.02$ (12.5%); c= $p<0.01$ (15.6%); d= $p<0.0001$ (16.7%). (B) BW on PND11. Significant treatment effect by ANOVA (LSM) and reduction in BW; different from control a= $p<0.001$ (25%); b= $p<0.003$ (23.8%); c= $p<0.001$ (23.9%); d= $p<0.01$ (13%).

CHAPTER 6

ATRAZINE AND EXPOSURE PARAMETERS WITHIN CRITICAL PERIODS OF DEVELOPMENT

Summary

The previous chapters examine the effects of gestational versus residual lactational atrazine (ATR) exposure and the critical three-day window of susceptibility in mammary gland development. The studies reported here evaluate the exposure parameters within each critical window to determine if residual lactational ATR exposure plays a role in the observed early mammary gland developmental delays.

Methods

Animals-Time-pregnant Long Evans rats (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed in an AAALAC accredited facility, one per cage and given food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. They were maintained in a room with a 14:10 hour light cycle, 20-24°C and relative humidity of 40-50%. Animal protocols were reviewed and approved by the National Health and Environmental Effects Research Laboratory, Institutional Animal Care and Use Committee.

Dosing Solution and Procedures- ATR (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. Timed-pregnant rats were treated in the morning with 0 (vehicle) or 100 mg ATR/kg BW by oral gavage in 5 ml/kg dosing volume. This ATR reference dose (100 mg/kg/d) was chosen due to consistent reproductive endpoint effects observed in previous studies (Laws *et al.*, 2000; Rayner *et al.*, 2004; Stoker *et al.*, 1999).

Experimental Design -Fifty pregnant LE dams were treated with vehicle (control) or 100 mg/kg atrazine once daily (N=25 dams/treatment). Control and ATR dams were dosed on either gestational days (GD) 13-19, 13-15, 15-17, or 17-19. On postnatal day 1 (day of birth), litters were weighed, equalized to 10 pups, and each full litter was cross-fostered with another litter from a control (C) or atrazine treated (ATR) dam, creating 10 exposure parameters (exposed dam-milk source; C-C GD13-19, ATR-C 13-19, C-ATR13-19, ATR-C 13-15, C-ATR13-15, ATR-C 15-17, C-ATR15-17, ATR-C 17-19, C-ATR17-19, and ATR-ATR13-19).

Necropsy- Necropsies were performed following an overnight and continued stay in a quiet holding area, and by using decapicones for animal transfer to reduce stress. All dams, female, and male pups were sacrificed on PND4.

Mammary Whole Mounts- The 4^{th} and 5^{th} mammary glands of female offspring were removed, fixed, and stained in carmine alum as a whole mount as previously described (Fenton *et al.*, 2002) on PND4. Flattened whole mounts were visualized and the epithelial outgrowth was measured to the closest millimeter (mm). Width measurements for area were taken from the two longest points of outgrowth. The whole mounts were subjectively scored (scale=1-4; 1=poor development/structure and 4=normal development/structure for each age group) within an age group, by two individual scorers without knowledge of treatment.

Statistical Analysis- Dam or litter means were calculated for body weights and mammary gland scores. Body weights and mammary gland scores were evaluated for treatment effects within each age group by one-way analysis of variance (ANOVA, Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC). Analysis of covariance, with body weight as a covariate, was used to evaluate the effects of treatment on mammary gland scores. Significant treatment effects were demonstrated by $p<0.05$ and specific p-values are indicated throughout.

Results

Dam Weight Gain- As observed in previous studies, all dams exposed to ATR failed to gain as much weight as control dams. Weight gain between control and treated dams was similar when treated dams were not being dosed with ATR (Table 6.1). During their respective dosing periods ATR GD13-15 gained 3.9 $\pm$ 2.6 g. ATR GD15-17 gained 5.3 $\pm$ 10.3g, ATR GD17-19 gained 12.7 $\pm$ 4.6 g, and ATR GD13-19 gained 41.1 $\pm$ 4.1g. All were significantly different when compared to control, $p<0.002$ and when compared to control cohorts. The lack of weight gain significantly reduced their total weight gain over the seven day period, $p<0.02$.

Growth of offspring- The differences in dam weight gain did not affect litter size or weights on PND1 (Table 6.2), but the ATR litters tended to weigh less than control litters and have slightly fewer pups. However, the average weight per pup was similar among all treatment groups. Differences in weight were noted in some females born to ATR dams at PND4 (Table 6.3). Females in the ATR-C GD13-15, ATR-C GD13-19, and ATR-ATR GD13-19 groups, had statistically different weights (6.7, 8.6, and 8.5% of control, respectively).

Offspring mammary gland development- Mammary glands were removed from offspring at PND4, scored for development, and measured as described in Chapter 5 (Table 6.4). All offspring born to an ATR-treated dam displayed delayed mammary gland development in the fourth and fifth mammary gland (ATR-C and ATR-ATR groups). They received lower scores based on lateral branching off of the primary ducts and branches and the inappropriate absence of terminal buds. Females born to ATR dams displayed decreased branching and decreased number of terminal buds. Females nursing from ATR dams dosed either GD17-19 or GD13-19 also consistently scored significantly lower in development than controls. Their

glands were similar in appearance to those of ATR born offspring in that they displayed moderate branching and budding. The fourth gland seems to be most sensitive to ATR effects as glands of animals born to a control dam and nursed from an ATR dam treated GD13-15 and GD17-19 were smaller in area compared to control glands (Table 6.4). However, the lateral and longitudinal growth effects of those treatments were not seen in the fifth mammary gland. Mammary glands from offspring born to ATR dams treated GD15-19 were significantly smaller in area in the fourth and fifth glands. These data suggest that the critical time of ATR exposure for effects on the developing MG are *in utero*. The effects, judging by MG developmental scores and size of the glands are worse as the ATR exposure gets closer to the time of parturition.

Discussion

The results of these studies demonstrate that a brief exposure to 100 mg/kg ATR/d during the latter part of gestation only is enough to delay mammary gland development in the female offspring regardless of the milk source. The results also suggest a residual lactation effect of ATR if dams were dosed during GD17-19. The glands of the offspring nursed by an ATR treated dam dosed during GD17-19 (including dams dosed GD13-19) were delayed in development. These results confirm that gestational ATR exposure does not alter litter weights at birth, and caused inconsistent decreases in weight on PND4. Only the earliest three day (GD13-15) and longest seven day (GD13-19) exposure was associated with decreased weight at PND4, but only in female offspring.

Mammary gland development in the offspring begins during the latter part of gestation and isometric growth of the gland continues until puberty (Imagawa et al., 1994; Sakakura, 1987). The gestational ATR exposure in these studies coincided with different developmental events of fetal mammary gland development (bud formation, differentiation, and epithelial proliferation) and appeared to act directly on the mammary gland during those events. The mammary gland effects of the *in utero* exposure were not rescued by milk from a control dam suggesting that the gestational exposure (in all exposure groups) caused permanent changes to the developing fetal mammary gland. The most severe delays were observed in animals exposed for the entire seven day period suggesting a cumulative or additive effect from continued exposure.

Early postnatal mammary gland development is characterized by epithelial branching and growth into the fat pad (Sakatura, 1987; Imagawa et al., 1994). The offspring nursed from GD17-19 and GD13-19 ATR treated dams displayed reduced branching and

differentiation. These animals were only exposed through lactation, adding evidence to the idea ATR alters the amount of parent compound and/or metabolites found in milk that can be transferred to the pup or cause a change in milk composition (i.e. lower protein or fat levels).

The results support the preceding chapter's evidence that maternal /fetal weight gain and early pup weight gain do not play a role in offspring mammary gland development. When exposed to 100 mg/kg ATR, dams do not gain as much weight at control dams, and it appears that ATR may act as an appetite suppressant. Because, when exposure ended, in the case of the three day exposure, weight gain approached that of controls. Weight gain in the GD13-19 dams began to approach controls after the first three days of dosing suggesting that over time the animals were able to cope with the exposure. The lack of weight gain had no effect on the offspring. The body weights of the offspring were not different when the pups were born and body weight did not influence mammary gland development at PND4.

Acute *in utero* exposure to 100 mg/kg ATR alone consistently delays offspring mammary gland development. In addition to the effects ATR appears to have transplacentally, in the dam, late gestational ATR exposure (specifically exposures occurring after GD17) may cause changes in the milk that directly affect the female offspring mammary glands or may additionally expose the pups to residual ATR metabolites in the milk. These effects occur when dams are dosed GD17-19 suggesting that this is the sensitive window of fetal mammary gland development as well as dam milk production/transference.

Table 6.1. Effect of 100 mg/kg ATR on Dam Weight Gain.

Group	N	GD13-15	GD15-17	GD17-19	GD13-19*
GD13-19 C	9	24.6 ± 2.1	39.2 ± 2.5	43.0 ± 2.6	67.6 ± 3.5
GD13-15 C	5	33.3 ± 2.6	39.6 ± 3.7	39.4 ± 3.8	72.7 ± 6.4
GD15-17 C	5	21.3 ± 4.7	28.1 ± 8.2	38.3 ± 10.5	59.6 ± 15
GD17-19 C	6	30.2 ± 3.0	42.7 ± 1.7	44.6 ± 1.7	74.9 ± 3.6
GD13-15 A	5	3.92 ± 2.6[a]	40.8 ± 3.9	40.4 ± 3.4	44.3 ± 4.3[b]
GD15-17 A	4	5.50 ± 5.6[a]	5.32 ± 10.3[a]	27.4 ± 9.7	32.9 ± 15[b]
GD17-19 A	6	26.5 ± 3.6	20.1 ± 5.7[a]	12.7 ± 4.6[a]	39.2 ± 7.0[a]
GD13-19 A	9	7.74 ± 2.3[a]	30.5 ± 3.6	33.3 ± 2.4	41.1 ± 4.1[a]

ATR= atrazine, GD= gestation day. Data presented as dam mean ± SE. * Accumulating weight gain with overlap. Significant treatment effect by ANOVA, [a] p<0.01 vs. GD13-19 C and control cohort C, [b] p<0.05 vs. GD13-19 C and control cohort C.

Table 6.2. Effect of 100 mg/kg ATR on Litter Weight and Size at PND1.

Group	N	Litter Weight (g)	Ave # of Pups	Weight per Pup
GD13-19 C	8	75.5 ± 3.3	13.5 ± 0.80	5.7 ± 0.27
GD13-15 C	5	74.5 ± 5.9	14.0 ± 1.0	5.3 ± 0.03
GD15-17 C	4	79.6 ± 3.3	14.5 ± 0.50	5.5 ± 0.17
GD17-19 C	6	73.9 ± 2.9	13.5 ± 0.62	5.5 ± 0.13
GD13-15 A	5	69.8 ± 4.0	12.8 ± 0.86	5.5 ± 0.15
GD15-17 A	3	67.7 ± 2.5	12.3 ± 0.67	5.5 ± 0.22
GD17-19 A	6	69.2 ± 4.1	12.8 ± 0.75	5.4 ± 0.17
GD13-19 A	8	71.3 ± 3.0	12.9 ± 0.64	5.6 ± 0.12

ATR= atrazine, GD= gestation day, PND= postnatal day. Data presented as dam mean ± SE.

Table 6.3. Effect of 100 mg/kg ATR on Offspring Weight at PND4.

Group	Female	Male
GD13-19 C-C[1]	8.80 ± 0.18	8.75 ± 0.46
GD13-15 C-ATR	8.78 ± 0.14	9.17 ± 0.39
GD15-17 C-ATR	9.55 ± 0.16	9.53 ± 0.32
GD17-19 C-ATR	9.63 ± 0.12	9.99 ±0.18
GD13-19 C-ATR	8.80 ± 0.24	9.34 ± 0.39
GD13-15 ATR-C	8.21 ± 0.16[a]	8.93 ± 0.18
GD15-17 ATR-C	8.20 ± 0.27	8.49 ± 0.27
GD17-19 ATR-C	8.52 ± 0.17	8.90 ± 0.21
GD13-19 ATR-C	8.04 ± 0.18[b]	8.16 ± 0.18
GD13-19 ATR-ATR	8.05 ± 0.46[c]	8.86 ± 0.22

ATR= atrazine, PND= post natal day. Data presented as dam mean ± SE. [1]Dam-Milk source. Significant treatment effect by ANOVA, [a] $p<0.05$ vs. C-C, [b] $p<0.01$ vs. C-C, [c] $p<0.02$ vs. C-C.

Table 6.4. Effect of 100 mg/kg ATR on Mammary Glands and Area (mm^2) at PND4.

Group	Gland 4 Score	Gland 4 Area	Gland 5 Score	Gland 5 Area
GD13-19 C-C[1]	3.1 ± 0.3	9.1 ± 0.6	3.1 ± 0.2	8.7 ± 0.7
GD13-15 C-ATR	2.7 ± 0.1	6.7 ± 0.5[a]	2.9 ± 0.1	7.5 ± 0.5
GD15-17 C-ATR	3.3 ± 0.1	9.6 ± 0.7	2.8 ± 0.1	9.0 ± 0.8
GD17-19 C-ATR	2.5 ± 0.1[a]	6.6 ± 0.5[a]	2.4 ± 0.1[a]	7.8 ± 0.6
GD13-19 C-ATR	2.3 ± 0.2[a]	7.5 ±0.7	2.5 ± 0.2[a]	8.3 ± 1.0
GD13-15 ATR-C	2.4 ± 0.1[a]	6.0 ± 0.4[a]	2.3 ± 0.1[b]	7.1 ± 0.6
GD15-17 ATR-C	2.1 ± 0.2[a]	5.7 ± 0.6[a]	2.1 ± 0.1[a]	6.1 ± 0.4[a]
GD17-19 ATR-C	2.5 ± 0.1[a]	6.1 ± 0.1[a]	2.2 ± 0.1[b]	5.7 ± 0.4[a]
GD13-19 ATR-C	1.8 ± 0.1[b]	4.4 ± 0.4[b]	1.8 ± 0.1[b]	6.0 ± 0.5[a]
GD13-19 ATR-ATR	1.9 ± 0.1[b]	5.1 ± 0.6[b]	1.7 ± 0.1[b]	4.7 ± 0.6[b]

ATR= atrazine, PND= post natal day. Data presented as dam mean ± SE. [1]Dam-Milk source. Significant treatment effect by ANOVA,[a] p<0.01 vs. C-C, [b] p<0.0001 vs. C-C.

CHAPTER 7

ATRAZINE ALTERS DAM MAMMARY GLAND DEVELOPMENT

Summary

Atrazine (ATR) was shown to delay early mammary development in female rat offspring following a brief gestational exposure. ATR can also cause developmental effects via nursing, as shown in Chapters 4 and 6. Because it is not known if the lactational effects of ATR are due to changes in quality, amount of milk transferred, or to actual ATR exposure, I hypothesized that ATR would adversely affect pregnancy-related dam mammary gland differentiation. On the contrary, the studies presented here test this hypothesis and suggest that exposure to ATR during pregnancy causes precocious dam mammary gland development. The proper development of dam mammary glands during pregnancy is very important to the well-being of the offspring and could play a role in the delayed mammary gland development observed in the offspring.

Methods

Animals-Time-pregnant Long Evans rats (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed in an AAALAC accredited facility, one per cage and given food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. They were maintained in a room with a 14:10 hour light cycle, 20-24°C and relative humidity of 40-50%. Animal protocols were reviewed and approved by the National Health and Environmental Effects Research Laboratory, Institutional Animal Care and Use Committee.

Dosing Solution and Procedures- Atrazine, ATR, (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. The environmentally based mixture (EBM) was prepared as a suspension in 1.0% methyl cellulose (Sigma) in distilled water. The EBM is a mixture of atrazine and its metabolites developed from the data accumulated from the North Carolina State Extension office and based on the highest measured levels of atrazine metabolites. It consists of atrazine (25%), 2-chloro-4, 6-diamino-s-chlorotriazine (DACT 35%), hydroxyatrazine (HA 20%), 2-chloro-4-amino-6-(isopropylamino)-s-triazine (DEA 15%), and 2-chloro-4-amino-6-(ethylamino)-s-triazine (DIA 5%). Time pregnant rats were treated in the morning with 0 (vehicle), EBM (total concentration equal to 8.7 mg/kg or 250 ppm), or 100 mg atrazine/kg BW by oral gavage in 5 ml/kg dosing volume. This reference dose (100 mg/kg/d) was chosen due to consistent reproductive endpoint effects observed in previous studies (Stoker *et al.*, 1999, Laws *et al.*, 2000, Rayner *et al.*, 2004).

Experimental Design- One hundred two pregnant LE dams were treated with vehicle (control), 8.7mg/kg EBM, or 100 mg/kg ATR daily (N>24 dams/ exposure period/treatment). Control and EBM dams were dosed gestational days (GD) 13-19, and dams receiving ATR

were dosed either GD17-19, or GD13-19. Dams and litters were sacrificed on GD20 (N= 5 dams/group), GD21 (N≥ 4 dams/group), PND1 (N=5 dams/group), PND4 (N≥5 dams/group), and PND11 (N= 4 dams/group). On PND4, remaining litters were weighed and randomly equalized to 10 pups (6 females, 4 males). On PND11, dams and pups were lactationally challenged (Appendix A). Dams and pups were sacrificed immediately after challenge. .

Necropsy- Necropsies were performed following an overnight stay in a quiet holding area and by using decapicones for animal transfer to reduce stress. At all necropsies, trunk blood was collected and portions of the 4th and 5th mammary glands of each dam were removed and processed for RNA and histology. For RNA, mammary gland was placed in 1 mL Tri Reagent (Sigma). Glands were placed in histology cassettes in 10% buffered formalin. These glands were sectioned and stained with hemotoxylin and eosin for developmental scoring.

On GD20 and 21, dams were decapitated, and trunk blood was collected and centrifuged for 30 minutes at 3000rpm (4°C) for serum. The uterus of each dam was removed and opened. Fetuses were counted and decapitated. On PNDs1 and 4, dams were sacrificed and treated as above. The uterus of each dam was removed and implantation and resorption sites were counted. The weight and sex of each pup was recorded and the pups discarded after mammary gland removal from female offspring. Remaining litters were culled to 10 pups.

Mammary Whole Mounts- The 4th and 5th mammary glands were removed from pups, fixed, and stained in carmine alum as a whole mount as previously described (Fenton *et al.*, 2002) on PNDs4 and 11. Flattened whole mounts were visualized and the epithelial outgrowth was

measured to the closest millimeter (mm). Length measurements for area were taken from the nipple to the farthest point of branching. Width measurements for area were taken from the two longest points of outgrowth.

Mammary Gland Scoring- Dam mammary glands and offspring whole mounts were subjectively scored (scale=1-4; 1=poor development/structure and 4=normal development/structure for each age group; operating procedure available upon request) within an age group, by two individual scorers without knowledge of treatment. For dam developmental scores, a higher score was given to advanced development of the gland. Dam mammary glands were also scored for ductal dilation and lipid accumulation in the same manner. Mammary glands representative of the mean score of the group were photographed on a Leica WILD M420 macroscope.

Radioimmunoassay- Serum was obtained from dams that were decapitated on GDs 20, 21, PNDs 1, 4, and 11 for use in radioimmunoassay. Serum corticosterone and progesterone were measured using Coat-a-Count Radioimmunoassay Kits obtained from Diagnostic Products Corporation (Los Angeles, CA). Serum estrone was measured using the DSL 8700 Estrone Radioimmunoassay kit from Diagnostic Systems Laboratories, Inc. (Webster, TX). Serum prolactin (PRL) was analyzed by radioimmunoassay using materials supplied by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases. All assays were run in duplicate.

Statistical Analysis- Group means and dam means (pups/dam; litter as unit) were calculated for body weights, mammary gland scores, and serum hormones and were evaluated for treatment effects within each age group by one-way analysis of variance (ANOVA, Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC). Analysis of covariance

was used to evaluate the effects of treatment on mammary gland scores while adjusting for any effect of body weight. Significant treatment effects were indicated by $p<0.05$ and specific p-values are indicated.

Results

Dam weight gain- As seen in our previous studies, dam weight gain during dosing in the ATR group was reduced when compared to weight gain of dams from the control and EBM groups. From GD13-17, control, EBM, and ATR GD17-19 (dosed only one day during this period of time) dams gained a similar amount of weight (~38.9 grams, g). Dams in ATR GD13-19 group averaged a gain of -2.6 $\pm$ 6.9 g ($p<0.0001$) over the same time period. The ATR GD17-19 dams were dosed starting on GD17 and gained an average of 5.2 $\pm$ 2.2 g ($p<0.0001$) during their three day dosing period, while dams in the remaining groups gained an average of 27 grams during this period of dosing. Throughout the entire dosing period, GD13-19, both GD17-19 and GD13-19 ATR groups, gained significantly less weight than control dams (66.5 $\pm$ 2.2 g); 44.7 $\pm$ 3.8 g ATR GD17-19 and 24.4 $\pm$ 6.7 g ATR GD13-19 (both $p< 0.001$). This reduced weight gain did not affect litter size as the number of pups per litter was not different in any of the groups on GD20, GD21, or in the number of implantation sites at PND1, 4, or 11. Following dosing, the remaining dam weights were recorded at PND 1 and found not to be different among the groups. At PND4, dams in the ATR GD13-19 groups weighed significantly less than control dams ($p<0.02$, 265.2 $\pm$ 8.2 g ATR GD13-19 vs. 303.5 $\pm$ 9.3 g Control). By PND11, dam weights between the dose groups were no longer different.

Dam serum hormone concentration- To determine if ATR exposure affected circulating hormone levels during this developmentally important period, serum was separated from trunk blood of dams on GD20, 21, PND1, 4, and 11 (Table 7.1). Progesterone, corticosterone, estrone, and prolactin were chosen because of their role in mammary gland development, pregnancy maintenance, and lactation. At GD20, progesterone levels were

found to be significantly lower in ATR GD17-19 dams ($p<0.02$ vs. Control). No other hormones were found to be statistically different at this time or any other timepoint, but interesting trends were noted among treatment groups. However, progesterone levels in ATR GD17-19 remained low at GD21. Corticosterone levels remained high throughout the end of pregnancy and early lactation and lowered by PND11 except in the two ATR groups. Estrone levels were higher in the two ATR groups the day before parturition and remained high after birth. Prolactin levels were similar across the groups. Hormone ratios were not found to be different (date not shown).

Dam mammary gland development- Dam mammary glands were removed on GD20, 21, PND1, 4, and 11 and scored for development, ductal dilation, and lipid accumulation (Table 7.2, Figures 7.1-7.5). Developmental scoring was based on how much of the gland had filled with lobuloalveolar units and the size of the units. Advanced or precocious development, different from controls ($p<0.004$), was observed in the glands of ATR GD13-19 dams at the first two timepoints. Glands from ATR GD17-19 were only statistically different at GD21, $p<0.005$, and developmentally, glands from EBM were not different from controls at any timepoint. Following parturition, development was not statistically different from controls in ATR GD13-19.

Ductal dilation was characterized by enlarged ductal structures occurring throughout the gland. This was not expected and highly abnormal. Oddly, glands from ATR GD13-19 dams had a significant increase in ductal dilation than controls at the first three timepoints, $p<0.003$ (Figures 7.1-7.3). Strangely, ATR GD17-19 and EBM also displayed these abnormal ductal and were scored significantly different from controls at GD21 and PND1, $p<0.02$. None of the groups were different from controls at PNDs 4 and 11 (Table 7.2,

Figures 7.4-7.5). There was an increased amount of lipid in the lobuloalveolar structures in the ATR GD13-19 glands at GD20, GD21, and PND1 ($p<0.02$), that was consistent with precocious development. Coincident with abnormal ductal dilation and precocious development on GD21, lipid accumulation in the ATR GD17-19 and EBM glands were different at GD21, $p<0.002$, but not at any other timepoint. Mammary glands from all treated groups displayed inflammatory cells within the ducts and in the glands at PND4 (Figure 7.4). Apoptotic bodies were noted in ATR exposed glands at PND11 (Figure 7.5).

Offspring Effects-

Offspring growth- Body weights were compared among the offspring of all groups at PND1, 4, and 11. At PND1, female pup weights were not different, but male pups from ATR GD17-19 weighed significantly less than control males ($p<0.003$, but only a 6.3% reduction). Consistent with our previous work (Chapter 4), females in both ATR groups weighed significantly less than control females by PND4 ($p<0.001$, 7.3% ATR GD17-19 and 9.1% ATR GD13-19). Their male siblings from the EBM group and both ATR groups weighed significantly less than control males ($p<0.02$, 4.8% EBM, 11.5% ATR GD17-19, and 8.1% ATR GD13-19). Only female offspring from ATR GD13-19 weighed significantly less than control at PND 11($p<0.001$, 11.6% reduction). Male offspring weights were not found to be different at this timepoint.

Offspring mammary gland development- Female offspring mammary glands removed on PNDs4 and 11 were scored for development and measured for epithelial area (Table 7.3). At PND4, glands from all groups were significantly reduced from control in development and size, $p<0.0001$. While all glands scored lower in development than control, there were some issues that separated each group. Glands from EBM offspring had a great amount of

variability, but the number and density of lateral branching was reduced consistently throughout all the glands. Lack of branching was an issue in the ATR GD17-19 glands and branching and budding were reduced in the ATR GD13-19 glands.

By PND11, developmental scores were still significantly lower than controls for all ATR-exposed groups, but the area of EBM and ATR GD17-19 glandular epithelium was not statistically different from control in the fourth gland or for EBM in the fifth gland. EBM glands still displayed density and branching detriments. ATR GD17-19 and GD13-19 had similar defects, with ATR GD13-19 having weak branching patterns and poor epithelial outgrowth.

Discussion

The results of the studies presented here demonstrate that gestational exposure to a low dose (8.7 mg/kg) mixture of ATR and its metabolites or to 100 mg/kg ATR/d altered development of the dams' mammary gland. As observed in previous studies and expected in these; mammary gland development was delayed in the offspring of the ATR treated dams as well as in the low dose mixture group. The delays were first observed at PND4, but remained until at least PND11.

We hypothesized that ATR would delay pregnancy-related mammary gland differentiation in a manner similar to that observed in the offspring leading to under-developed glands as pregnancy continued. In the offspring, ATR appears to act most directly when the glands are going through the epithelial proliferation stage which starts around GD17 and continues after birth. As the dam enters the final stages of pregnancy and begins to prepare for birth, the epithelial structures proliferate and differentiate to fill the fat pad and form lobuloalveolar structures (Masso-Welch *et al*., 2000). In this study, instead of delaying dam mammary gland development, ATR appeared to accelerate it especially in the GD13-19 ATR treated dams which filled the fat pad much earlier with lobulalveolar structures than controls. ATR exposure, either in low dose mixture or parent compound, also altered the ducts of the dam mammary gland by greatly increasing their size and distension within the gland. This occurred during late gestation through the day the pups were born. Coupled with the accelerated development, this ductile dilation suggests action on proliferating cells or active transfer of compound from the blood stream into the MG prior to beginning lactation.

The observed effects in the dam mammary gland occurred with only subtle changes in serum hormone concentrations. Hormones change very rapidly when transitioning from pregnant to lactating. With the exception of one group at one timepoint, we observed no differences in serum hormones. With the accelerated development and ductile dilation, we expected differences in estrone, progesterone, and prolactin concentrations as these are all known to be involved in lobuloalveolar differentiation and secretory development (Borellini and Oka, 1989; Imagawa *et al.*, 1994; Silberstein, 2001; Topper and Freeman, 1980). We did see corticosterone levels decrease following birth as pregnancy maintenance was no longer required. We also saw prolactin levels increase during mid-lactation due to the lactational challenge. Based on these data, the mammary gland alterations observed in the dams following exposure to ATR were not associated with changes in hormone concentrations.

As seen in the previous studies, offspring mammary gland development was delayed at early timepoints in all ATR exposed groups, even the low dose (8.7 mg/kg) mixture. Their developmental scores were very similar to the two ATR groups at PND4. This delayed development was mirrored by stunted area of the entire gland. By PND11, the 8.7 mg/kg EBM group had caught up to controls with respect to area of the gland but still displayed delayed development in the gland with scores only slightly higher than the two ATR groups. The mixture appears to be a very potent modulator of early mammary gland development.

In conclusion, gestational exposure to ATR, as well as a low dose mixture of ATR and its metabolites, not only delays offspring mammary gland development, but accelerates lactogenesis in the dam without affecting serum hormone concentrations. Therefore, these data suggest that decreased milk production and/or quality (lipid content) do not explain the mammary gland effects observed in females nursing from ATR-exposed dams. Further

studies evaluating milk transfer during a timed feeding and maternal behavior confirm this conclusion (Appendix B).

Table 7.1. Effect of 8.7 mg/kg EMB and 100 mg/kg ATR on Dam Serum Hormone Concentrations (/ml) Over Time.

GD 20	Progesterone (ng)	Corticosterone (ng)	Estrone (pg)	Prolactin (ng)
Control	51.6 ± 10	573 ± 116	42.9 ± 4.8	4.80 ± 1.7
EBM	26.6 ± 11	332 ± 70	29.6 ± 5.6	2.94 ± 0.36
ATR GD17-19	15.5 ± 6.6[a]	321 ± 72	45.6 ± 9.0	11.4 ± 7.1
ATR GD13-19	53.8 ± 9.7	371 ± 52	49.5 ± 10	4.26 ± 1.0
GD21				
Control	19.3 ± 7.9	517 ± 120	29.0 ± 3.5	60.4 ± 14
EBM	16.9 ± 7.2	674 ± 80.3	57.0 ± 18	63.7 ± 5.1
ATR GD17-19	8.79 ± 2.8	407 ± 137	53.1 ± 6.8	61.8 ± 11
ATR GD13-19	13.0 ± 3.1	453 ± 114	64.8 ± 24	42.2 ± 18
PND1				
Control	23.7 ± 6.1	639 ± 84	55.1 ± 6.6	5.81 ± 1.9
EBM	33.0 ± 5.4	554 ± 47	39.5 ± 5.2	5.63 ± 1.9
ATR GD17-19	34.4 ± 11	576 ± 46	167 ± 68	7.78 ± 2.4
ATR GD13-19	32.7 ± 11	452 ± 77	80.7 ± 34	4.24 ± 0.67
PND4				
Control	48.0 ± 8.8	437 ± 138	98.4 ± 22	6.65 ± 3.24
EBM	51.9 ± 6.4	498 ± 108	73.9 ± 12	4.34 ± 0.74
ATR GD17-19	62.8 ± 6.7	516 ± 87	148 ± 44	3.99 ± 1.0
ATR GD13-19	47.8 ± 8.1	407 ± 127	193 ± 110	3.73 ± 1.3
PND11				
Control	47.3 ± 8.4	51.0 ± 10	62.1 ± 12	127 ± 6.2
EBM	53.0 ± 12	82.6 ± 25	50.3 ± 2.8	109 ± 14
ATR GD17-19	68.5 ± 6.7	136 ± 14	105 ± 40	101 ± 12
ATR GD13-19	47.8 ± 12	110 ± 41	150 ± 39	128 ± 13

EBM= environmentally based mixture 8.7 mg/kg, ATR= atrazine, GD=gestation day, PND= post natal day. Data presented as dam mean ± SE. GD20, PND1- Dam N=5 per group, GD21- Dam N≥ 4 per group, PND4- Dam N≥ 5 per group, PND11- Dam N=4 per group. Significant treatment effect by ANOVA, [a] p<0.02 vs. Control.

Table 7.2. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on Dam Mammary Gland Development, Ductal Dilation, and Lipid Accumulation over Time.

Development				
Group	**Control**	**EBM**	**ATR GD17-19**	**ATR GD13-19**
GD20	**1.9 ± 0.1**	**2.1 ± 0.3**	**1.9 ± 0.4**	**3.4 ± 0.2***
GD21	**2.3 ± 0.2**	**2.6 ± 0.2**	**3.4 ± 0.2***	**3.5 ± 0.3***
PND1	**2.2 ± 0.4**	**2.7 ± 0.3**	**3.1 ± 0.3**	**3.4 ± 0.2***
PND4	**2.9 ± 0.4**	**2.4 ± 0.1**	**2.8 ± 0.3**	**3.4 ± 0.2**
PND11	**2.5 ± 0.2**	**3.2 ± 0.5**	**3.2 ± 0.4**	**3.2 ± 0.5**
Ductal Dilation				
GD20	**2.0 ± 0.3**	**2.6 ± 0.4**	**1.2 ± 0.2**	**3.6 ± 0.2***
GD21	**1.2 ± 0.2**	**3.2 ± 0.4***	**2.6 ± 0.4***	**3.0 ± 0.4***
PND1	**1.6 ± 0.2**	**2.8 ± 0.4***	**2.6 ± 0.2***	**3.4 ± 0.2***
PND4	**2.6 ±0.2**	**2.6 ± 0.3**	**2.7 ± 0.4**	**3.2 ± 0.4**
PND11	**2.5 ± 0.3**	**3.0 ± 0.0**	**3.0 ± 0.0**	**2.7 ± 0.2**
Lipid Accumulation				
GD20	**1.2 ± 0.2**	**1.8 ± 0.3**	**1.7 ± 0.25**	**3.6 ± 0.2***
GD21	**1.8 ± 0.2**	**3.2 ± 0.4***	**3.4 ± 0.2***	**3.7 ± 0.2***
PND1	**3.0 ± 0.0**	**3.2 ± 0.2**	**3.0 ± 0.3**	**3.8 ± 0.2***
PND4	**3.2 ± 0.2**	**2.7 ± 0.2**	**2.9 ± 0.3**	**3.6 ± 0.2**
PND11	**2.5 ± 0.2**	**2.5 ± 0.6**	**2.7 ± 0.2**	**3.7 ± 0.2**

EBM= environmentally based mixture 8.7 mg/kg, ATR= atrazine, GD=gestation day, PND= post natal day. Data presented as dam mean ± SE. GD20, PND1- Dam N=5 per group, GD21- Dam N≥ 4 per group, PND4- Dam N≥ 5 per group, PND11- Dam N=4 per group. Significant treatment effect by ANOVA, * p<0.02 vs. Control.

Table 7.3. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on Offspring Mammary Gland Development (4th and 5th gland) and Area (mm^2)

PND4	Control	EBM	ATR GD17-19	ATR GD13-19
Gland 4 Score	3.3 ± 0.1	2.4 ± 0.1*	2.4 ± 0.1*	2.3 ± 0.1*
Gland 4 Area	9.0 ± 0.4	6.5 ± 0.4*	5.5 ± 0.3*	6.6 ± 0.4*
Gland 5 Score	3.3 ± 0.1	2.2 ± 0.1*	2.3 ± 0.1*	2.1 ± 0.1*
Gland 5 Area	9.2 ± 0.5	6.4 ± 0.4*	6.0 ± 0.3*	6.0 ± 0.4*
PND11				
Gland 4 Score	3.5 ± 0.1	2.4 ± 0.2*	2.2 ± 0.2*	2.0 ± 0.2*
Gland 4 Area	20.4 ± 1.4	19.0 ± 2.7	15.7 ± 2.0	12.3 ± 2.1*
Gland 5 Score	3.6 ± 0.1	2.7 ± 0.2*	2.4 ± 0.2*	2.2 ± 0.2*
Gland 5 Area	18.9 ± 1.0	18.7 ± 1.7	12.9 ± 2.1*	12.3 ± 1.6*

EBM= environmentally based mixture, ATR= atrazine, PND= post natal day. Data presented as dam mean ± SE. PND4- Dam N≥ 5 per group, PND11- Dam N≥ 3 per group. Significant treatment effect by ANOVA, * p<0.01 vs. Control.

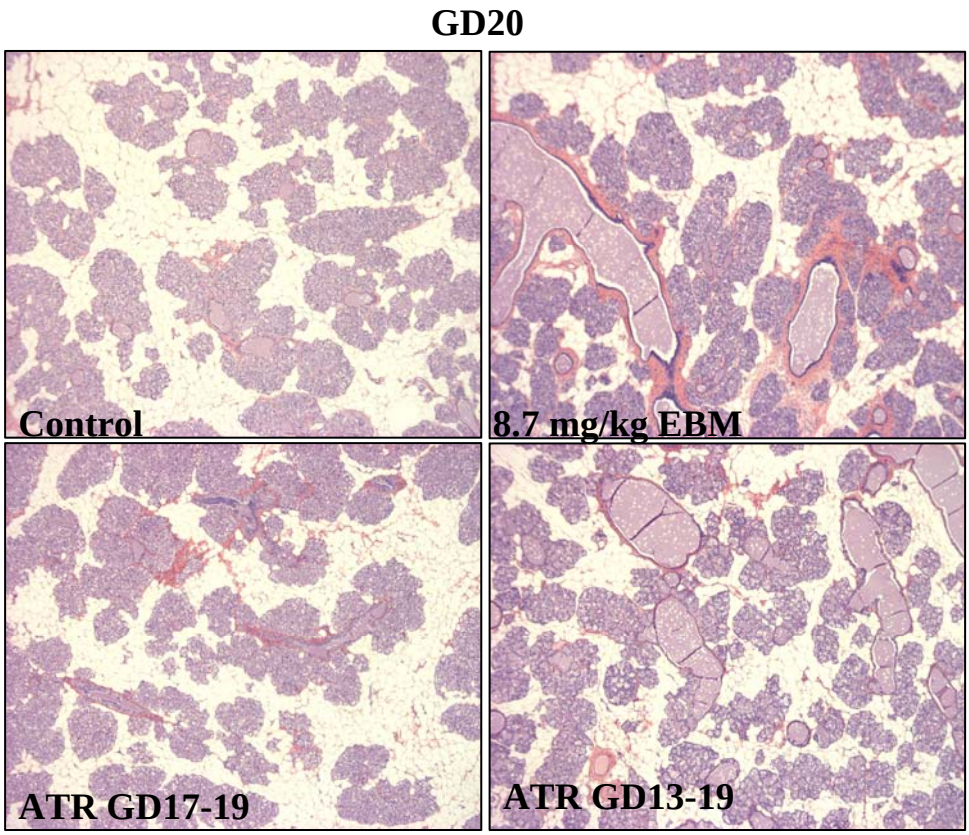


Figure 7.1. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on dam mammary gland development at GD20. ATR GD13-19 glands displayed increased development, ductal dilation, and lipid accumulation, $p<0.02$ vs. Control. 8.7 mg/kg EBM not statistically significant at this timepoint.

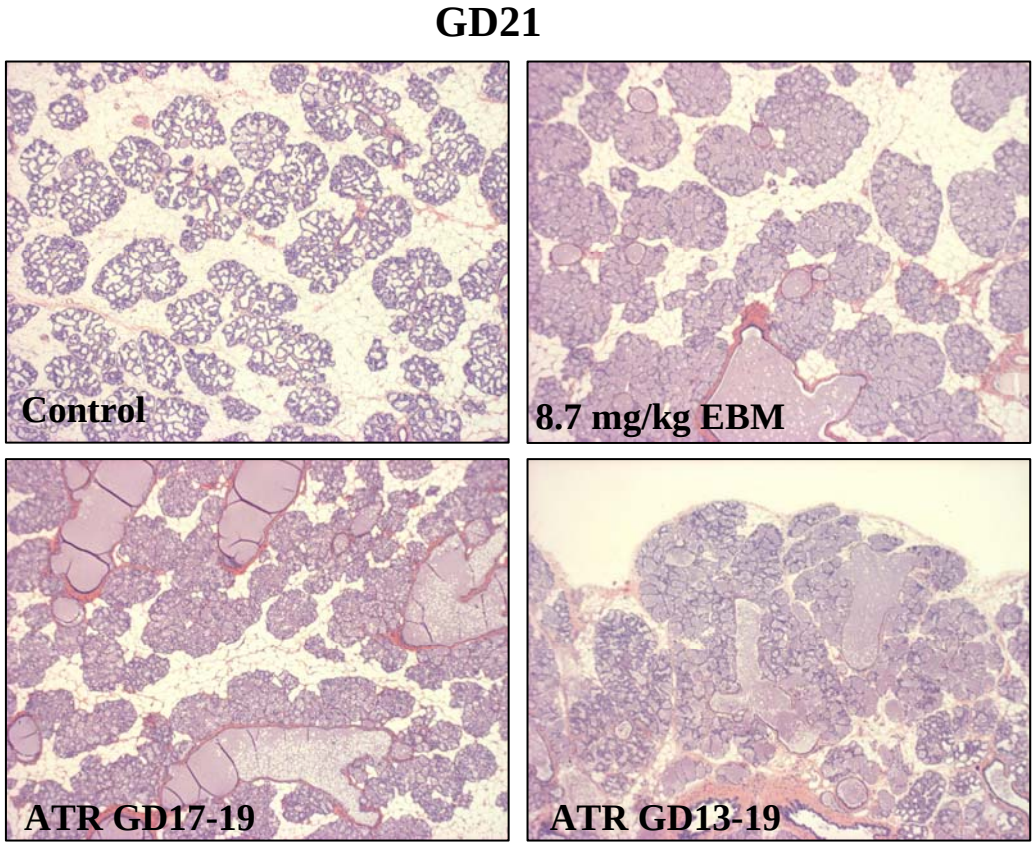


Figure 7.2. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on dam mammary gland development at GD21. All treated dam glands displayed increased ductal dilation, and lipid accumulation, $p<0.02$ vs. Control. ATR GD17-19 and GD13-19 scored higher in development than controls, $p<0.02$.

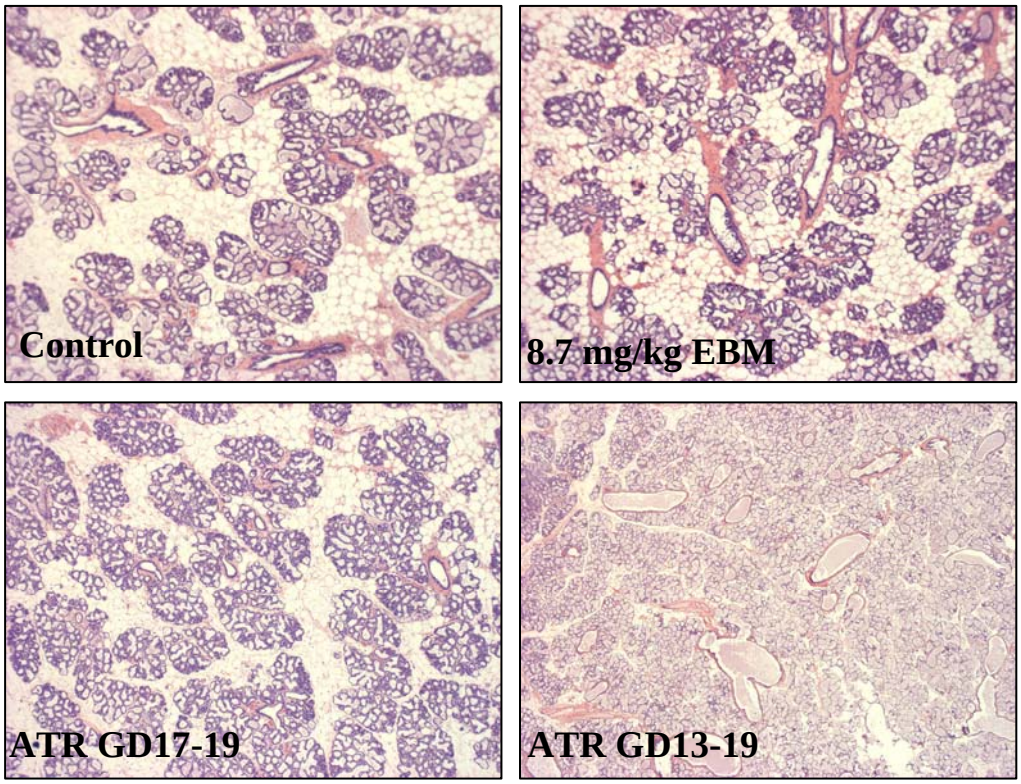


Figure 7.3. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on dam mammary gland development at PND1. All treated dam glands displayed increased ductal dilation. ATR GD13-19 had increased lipid accumulation at this timepoint, $p<0.02$ vs. Control and very little adipose was evident in glands of these animals.

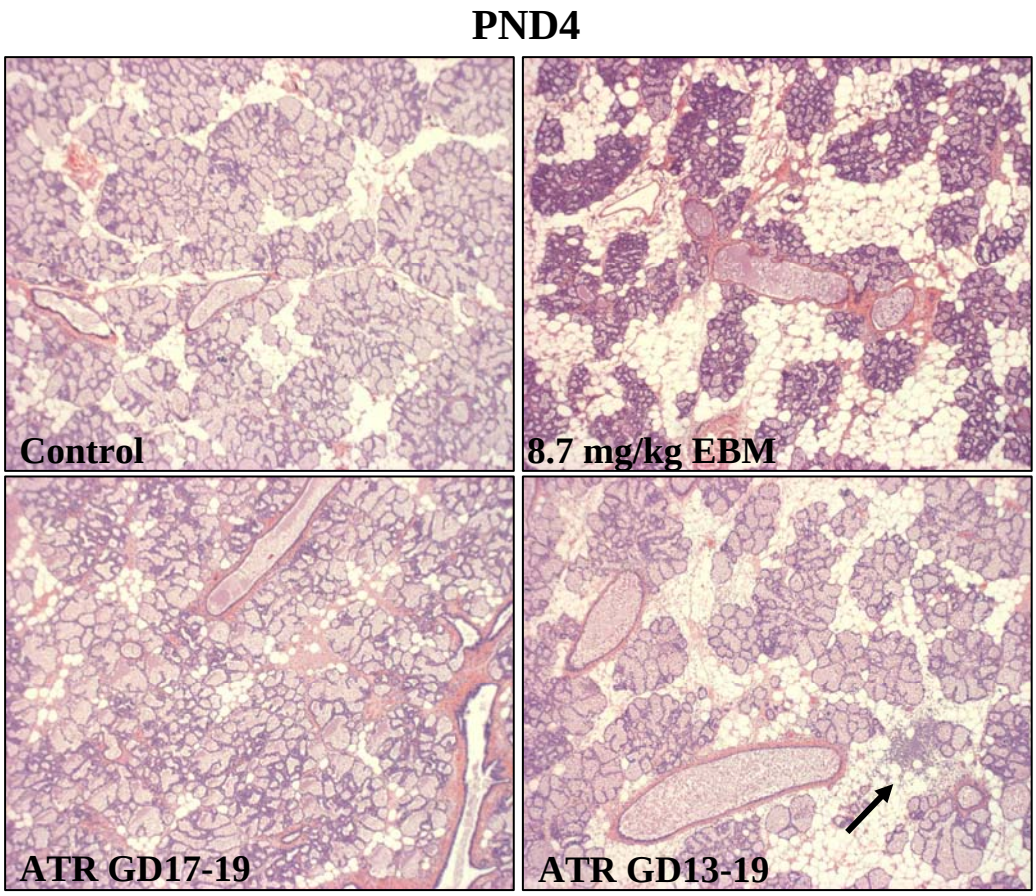


Figure 7.4. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on dam mammary gland development at PND4. No statistical differences were found for development, ductal dilation, and lipid accumulation. Ducts of control animals were now also filled with secretory materials. Inflammatory cells were found in the glands and ducts of the ATR treated animals (arrow).

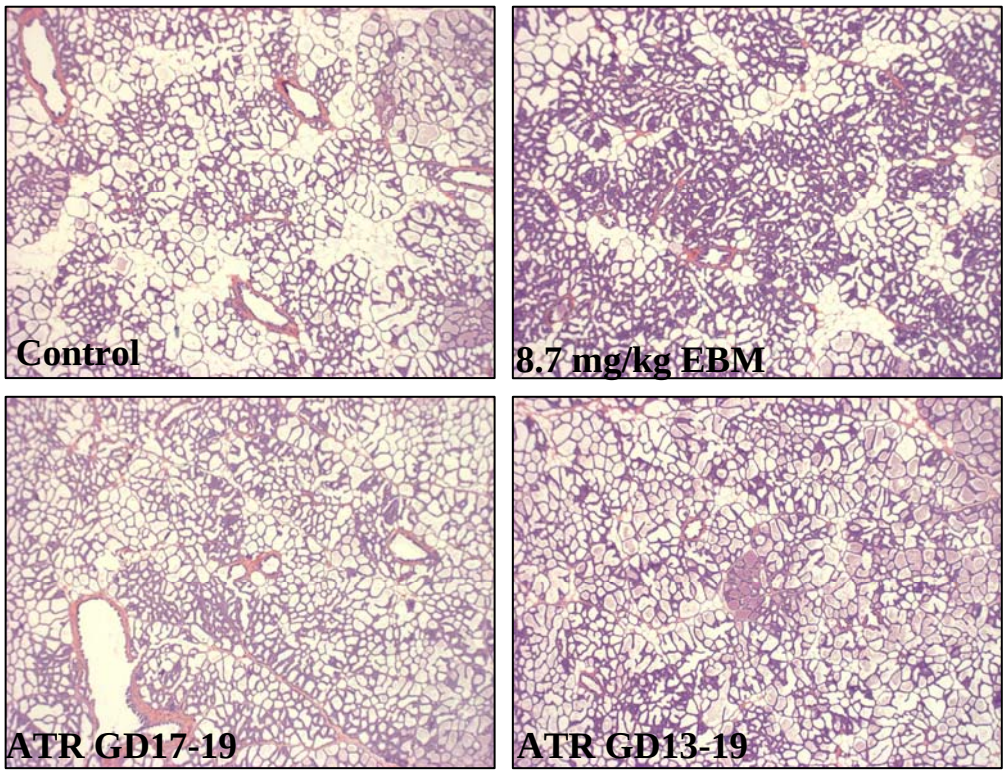


Figure 7.5. Effect of 8.7 mg/kg EBM and 100 mg/kg ATR on dam mammary gland development at PND11. No statistical differences were found for development, ductal dilation, and lipid accumulation. However, the fat pads appear to be totally filled with epithelium in treated animals when compared with control. Apoptotic bodies were noted in the glands of the ATR treated animals.

CHAPTER 8

DETECTION OF ATRAZINE IN THE PREGNANT AND LACTATING DAM

Summary

Atrazine (ATR) causes precocious lactogenesis and mammary gland development in Long-Evans dams following a brief gestation exposure as short as three days at 100 mg/kg or following a seven day low dose exposure (8.7 mg/kg). ATR is reported to have a half-life of less than 1 day in adult non-pregnant rats. However, control pups nursing from dams exposed to ATR have altered MG development and delayed puberty. We hypothesized that ATR disposition in the pregnant and lactating rat may differ from that in the adult non-pregnant rat resulting in residual ATR metabolite transference to pups both transplacentally and via milk. The studies presented here test this hypothesis and determine if ATR and its metabolites are found in milk, urine, serum, and amniotic fluid at different time points following dosing. The results showed that ATR and its metabolites can de detected in the dam at least 6 days post dosing. These results suggest that even though exposure to the pregnant dam ended on GD19, ATR metabolites may be available to the developing offspring until at least PND4 when abnormal mammary gland development has been detected in female pups, and neurological centers in the brain responsible for central nervous system control of puberty are suggested to be developing.

Methods

The animals and methods in this study were the same as used in the previous chapter except where noted below.

Experimental Design- A subset of dams were dosed with vehicle, 8.7 mg/kg EBM, or 100 mg/kg ATR on PND4 (exactly as they had been dosed prenatally) and sacrificed three hours post dosing. .

Milking and Urine Collection- On GD21, PND1, and PND4 dams were milked prior to sacrifice. Dams were injected with Oxytocin (5 IU/ml IM injection, Sigma). Dams were then injected with Ketamine:Xylazine (35:5 mg/kg IP injection). A milking device was created using the building's vacuum system, a side-arm flask with a one holed rubber stopper, and tubing from a commercial breast pump. Urine was collected from live animals five and 8 hours post dosing on GD19.

Necropsy- Necropsies were performed following an overnight stay in a quiet holding area and by using decapicones for animal transfer to reduce stress. At all necropsies, trunk blood was collected for serum and portions of the 4^{th} and 5^{th} mammary glands of each dam were removed and processed for extraction of ATR and metabolites. For tissue homogenate, mammary gland was weighed and placed in 1 mL 37% methanol and later homogenized and centrifuged to collect the supernatant. Urine from each dam was collected from the bladder with a syringe on GD20, 21, PNDs1, 4, and 11 at necropsy.

On GD20 and 21, dams were sacrificed after attempted milk collections. Dams (N=5) were decapitated, and trunk blood was collected and centrifuged for 30 minutes at 3000rpm (4°C) for serum. Uteri were removed intact and amniotic fluid from each dam at GD20 and 21 was collected, sonicated, and centrifuged to collect supernatant. On PNDs1, 4, and 11, dams

(N=5, PND1; N>7, PND4, N=5, PND11) were sacrificed and treated as above. Urine, amniotic fluid, serum, milk extract, and milk were sent to the Centers for Disease Control for ATR and metabolite detection.

Detection of Atrazine and Metabolites- Urine, amniotic fluid, and serum samples from control and ATR-dosed dams were analyzed by isotope dilution high performance liquid chromatography tandem mass spectrometry for the presence of hydroxyl-, dealkyl- and mercapturate ATR metabolites. The method can accurately quantify 10 ATR-related metabolites and qualitatively analyze two additional mercapturate metabolites with limits of detections ranging from 0.1-1 ng/ml. Values listed at 0 were below the limit of detection. The process was divided into three phases including sample loading, reverse phase separation and strong cation exchange separation and re-equilibration of reverse phase columns. Two hundred ul of sample were used and 2 ion pairs monitored for each metabolite. Detected metabolites include 2-chloro-4, 6-diamino-s-chlorotriazine DACT, hydroxyatrazine ATR-OH, 2-chloro-4-amino-6-(isopropylamino)-s-triazine DEA, and 2-chloro-4-amino-6-(ethylamino)-s-triazine DIA, and the mercapturate forms of ATR and DEA. The parent compound, ATR, was detected as well.

Results

Detection of ATR and/ or its metabolites

Urine- Urine was collected from exposed dams on GD19 as well as all the timepoints mentioned above. ATR and metabolites were detected in urine at all timepoints, including PND11 (Figures 1-4). There was very little ATR detected among the groups compared to what they were actually given orally, but ATR metabolites were still being excreted as long as 13 days post dosing. The major metabolites detected in EBM dams were ATR-OH $\geq$ DACT $\geq$ DEAM. In dams treated with ATR parent compound, DACT and DEAM were the major metabolites detected. During the last two days of gestation, the metabolite profile for ATR GD17-19 contained a fair amount of ATR-OH, but following birth, ATR-OH levels were very low.

All treated dams had a decrease in ATR and metabolites in the three hours between the collection periods on GD19 (Figures 8.1-8.3). A continued decrease was evident in urine of EBM treated dams on GD20, but a slight increase was observed in the dams on GD21 (Figure 8.1). Both ATR GD17-19 and GD13-19 showed an increase in detected components on GD20 and a decrease on GD21. Levels of metabolites increased on PNDs1 and 4 in the ATR GD13-19 dams followed by a decrease on PND11. Both EBM and ATR GD17-19 showed an increase in levels after parturition. It is not clear how these levels correlate with changes in creatinine in the urine. The reason for the large postnatal increase is unknown.

On PND4, a subset of dams were dosed with the compound they were prenatally exposed to and killed three hours later. Urine collected at that time demonstrated a dose-related increase in detected metabolite totals. ATR-OH was very similar to DACT in the EBM and DACT > DEAM in the dams dosed with the parent compound.

Amniotic fluid- To measure the ATR metabolites present, amniotic fluid was removed from dams at GD20 and 21, and fluid collected. DACT was the only metabolite detected in the amniotic fluid of EBM dams at GD20 and GD21 (very low levels). In the ATR GD17-19 dams, DACT was the major metabolite detected, but DIA, DEA, DEAM, and even the parent compound were also detected in lower concentations. ATR, ATRM, and ATR-OH were not detected in the ATR GD13-19 dams. From GD20 to GD21, DACT concentration in EBM females had decreased but was still detectable. ATR-OH and DIA were not detected in ATR GD17-19, but all forms were found in ATR GD 13-19 females on GD21.

Serum- Serum was analyzed at all timepoints for the presence of free ATR and its metabolites. Very little free metabolite was detected in serum relative to other body fluids evaluated. DACT was the major metabolite found in EBM dam serum on GD20. There was very little HA and DEA present and no other metabolites detected. DACT was the only metabolite detected at GD21 and PND1 in decreasing amounts. No serum analytes were detectable on PNDs4 and 11. Surprisingly, in both the ATR GD17-19 and GD13-19 groups, ATR-OH was the major available metabolite detected with DEAM, DIA, DACT, and DEA present at very low levels on GDs 20, 21, and PND1. By PND4, very little metabolite remained, but mostly ATR-OH was present in both groups. Although the levels were very low, ATR GD17-19 was the only group presenting any metabolites (ATR-OH and DEAM) on PND11.

A subset of dams was given one dose of vehicle, 8.7 mg/kg EBM, or 100 mg/kg ATR on PND4 and necropsied three hours later. The distribution of metabolites was similar to what was seen in animals dosed only during gestation. In the EBM dams, DACT was the major metabolite followed by ATR-OH, DEA and DIA. No DEAM or ATRM was detected.

In the ATR dosed dams, the metabolites order from greatest to least was ATR-OH, DEAM, DIA, and DACT. DEA was not detected in ATR GD17-19 but ATRM and ATR were found. DEA, ATRM, and ATR were detected in low amounts in ATR GD13-19 serum.

Discussion

The results of the studies presented here demonstrate that ATR and its metabolites can be detected in the urine, amniotic fluid, and serum of pregnant and lactating dams and is detectable six to thirteen days after exposure. Levels and ratios of analytes are different depending upon the starting exposure and exposure length. The results also demonstrated that the parent compound and a mixture of ATR + metabolites present different profiles in the fluids mentioned above.

Many studies have examined ATR in the virgin non-pregnant rats and found it to be rapidly eliminated (Atrazine Toxicology). The results of these studies suggest that this is not the case for the pregnant and lactating rat. In the urine, ATR and metabolites increased following birth of the offspring suggesting storage of the compounds during pregnancy and a subsequent release or a change in metabolism following parturition.

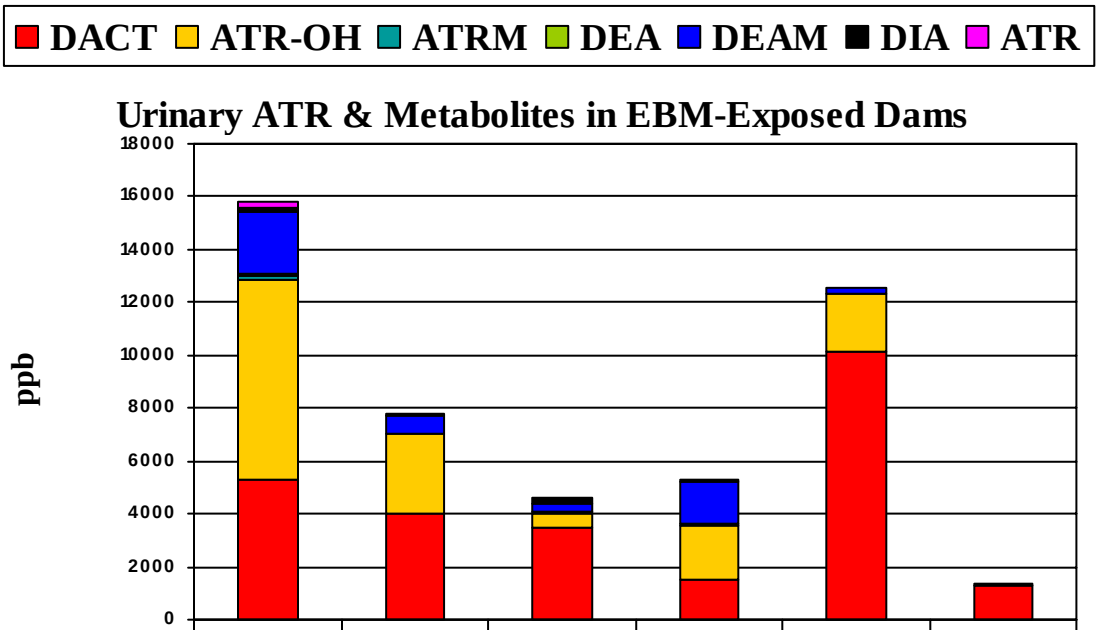
It is also possible that postnatal increase in levels of metabolites could be attributed to the maternal behavior of the dams. When the offspring are less than 10 days old, dams routinely groom them by licking their anogenital region (Smith *et al.*, 2004), to aid the pups in urination during this time. While doing this, the dams ingest urine and possible feces containing ATR metabolites.

In examining the profiles of urine, amniotic fluid, and serum in the exposed dams, we were surprised to observe that the amount of metabolites in the 8.7 mg/kg EBM dams were different from those found in the dams treated with only ATR. ATR-OH was found in relatively high levels in the urine, but was only 20% of the administered dose, suggesting that the metabolism of ATR-OH is slow. In amniotic fluid, DACT was the main metabolite detected in all groups, and it decreased over the two day period post dosing. However, this

data shows that the developing fetus is exposed to DACT and low amounts of other metabolites. Surprisingly, DACT in the EBM group, and ATR-OH in ATR treated group, were major metabolites found in serum, especially given that ATR-OH was a major metabolite in 8.7 mg/kg EBM dam urine.

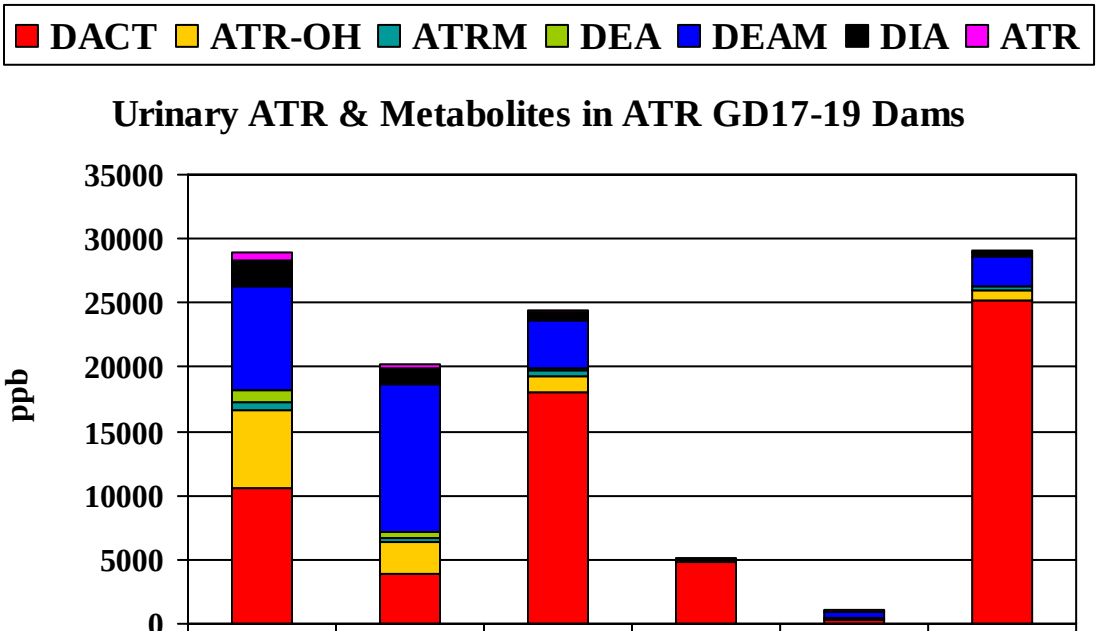
The metabolite profiles could be due to preferential binding of the metabolites to proteins or amino acid residues in the fluids in which they were detected (Dooley *et al.*, 2006; Wu *et al.*, 1998). Recent work by Dooley and colleagues (2006) identified a hemoglobin adduct in Sprague-Dawley rats exposed to ATR they speculate to be DACT. This could explain why the serum metabolite profiles of the two ATR groups were majority ATR-OH. It does not explain the DACT levels in the 8.7 mg/kg EBM dams. The different profiles could also be due to metabolism pathway saturation at the higher doses. Either way, these data demonstrate that ATR metabolites are available to the neonate via the exposed dam, both transplacentally as DACT or via the milk, which would recruit metabolites from blood.

In conclusion, ATR and its metabolites can be detected in urine, amniotic fluid, and serum many days post dosing in the pregnant and lactating rat. The data show that metabolites of ATR do reach the placenta, and potentially therefore the fetus. Dose and length of exposure appear to play a role in the distribution of the metabolites within each media tested. The difference in the ratio of metabolites may be the reason that EBM induces developmental delays in mammary glands of offspring at such low doses.



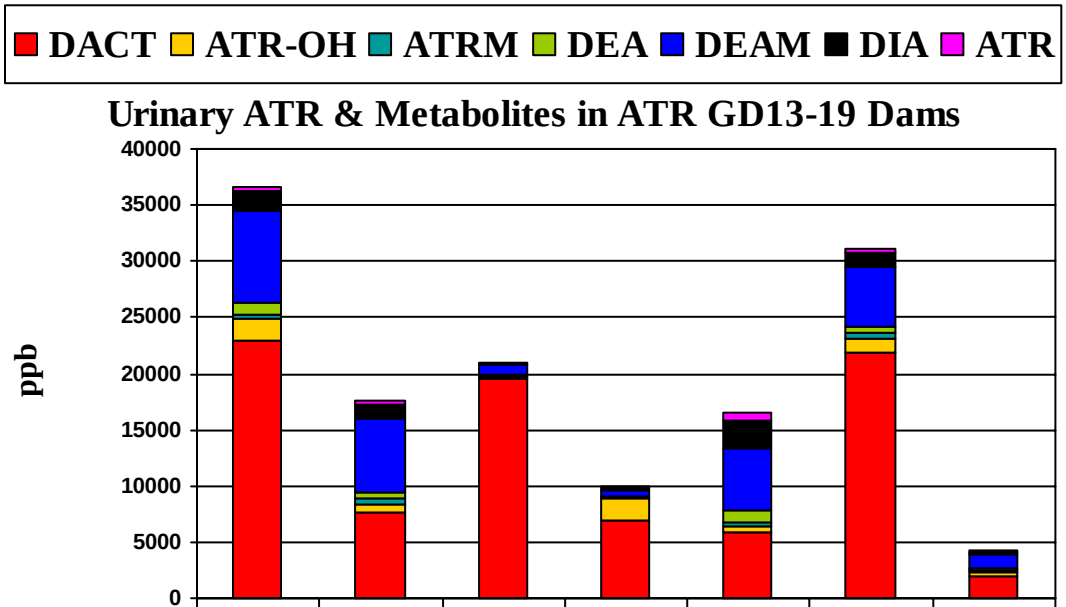
	GD19	GD19U	GD20	GD21	PND4	PND11
ATR	183	0	1	0	0	0
DIA	177	18	226	87	41	0
DEAM	2309	696	255	1634	214	0
DEA	134	8	59	24	11	0
ATRM	92	23	58	35	1.5	0
ATR-OH	7600	3009	503	2054	2154	138
DACT	5287	4007	3498	1483	10164	1252

Figure 8.1. Levels of ATR and metabolites in urine from 8.7 mg/kg EBM dams over time. Metabolites in GD19 and GD19U were detected from samples taken 5 and 8 hours post dosing. The major metabolites detected include ATR-OH and DACT.



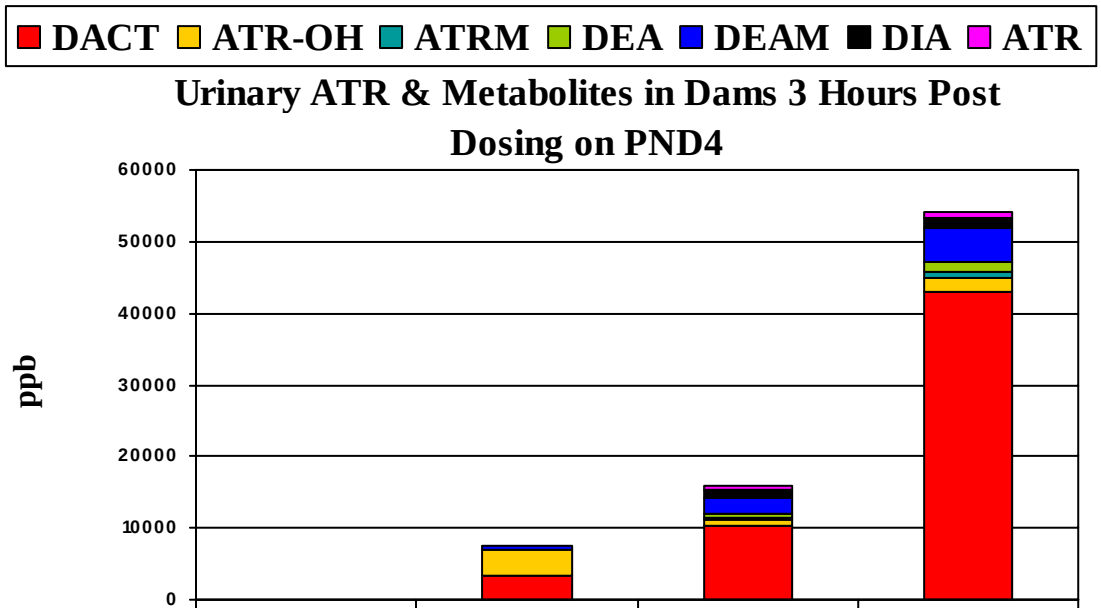
	GD19	GD19U	GD20	GD21	PND4	PND11
ATR	695	304	133	2	88	247
DIA	2028	1120	611	1.8	111	340
DEAM	8114	11491	3775	67	434	2305
DEA	826	493	205	23	35	28
ATRM	696	323	351	11	77	194
ATR-OH	5967	2569	1252	86	64	888
DACT	10635	3846	18077	4885	248	25156

Figure 8.2. Levels of ATR and metabolites in urine from ATR GD17-19 dams over time. Metabolites in GD19 and GD19U were detected from samples taken 5 and 8 hours post dosing. The major metabolites detected include DACT and DEAM.



	GD19	GD19U	GD20	GD21	PND1	PND4	PND11
ATR	479	320	32	18	769	458	96
DIA	1649	1388	151	324	2407	1249	192
DEAM	8215	6557	842	486	5505	5269	1287
DEA	1037	521	150	170	1051	639	198
ATRM	479	467	61	47	352	439	96
ATR-OH	1950	800	145	1911	523	1233.6	407
DACT	22854	7568	19545	6971	5934	21885	1926

Figure 8.3. Levels of ATR and metabolites in urine from ATR GD13-19 dams over time. Metabolites in GD19 and GD19U were detected from samples taken 5 and 8 hours post dosing. The major metabolites include DACT and DEAM.



	Control	EBM	GD17-19	GD13-19
ATR	0	1.6	580	719
DIA	0	74	1065	1595
DEAM	0	487	2485	4653
DEA	0	49	395	1342
ATRM	0	53	378	748
ATR-OH	0	3585	736	2173
DACT	0	3330	10375	42861

Figure 8.4. Levels of ATR and metabolites in urine three hours after dosing on PND4. Metabolites in GD19 and GD19U were detected from samples taken 5 and 8 hours post dosing. Levels of ATR-OH and DACT are similar in the EBM group, but DACT was the major metabolite in the ATR GD17-19 and GD13-19.

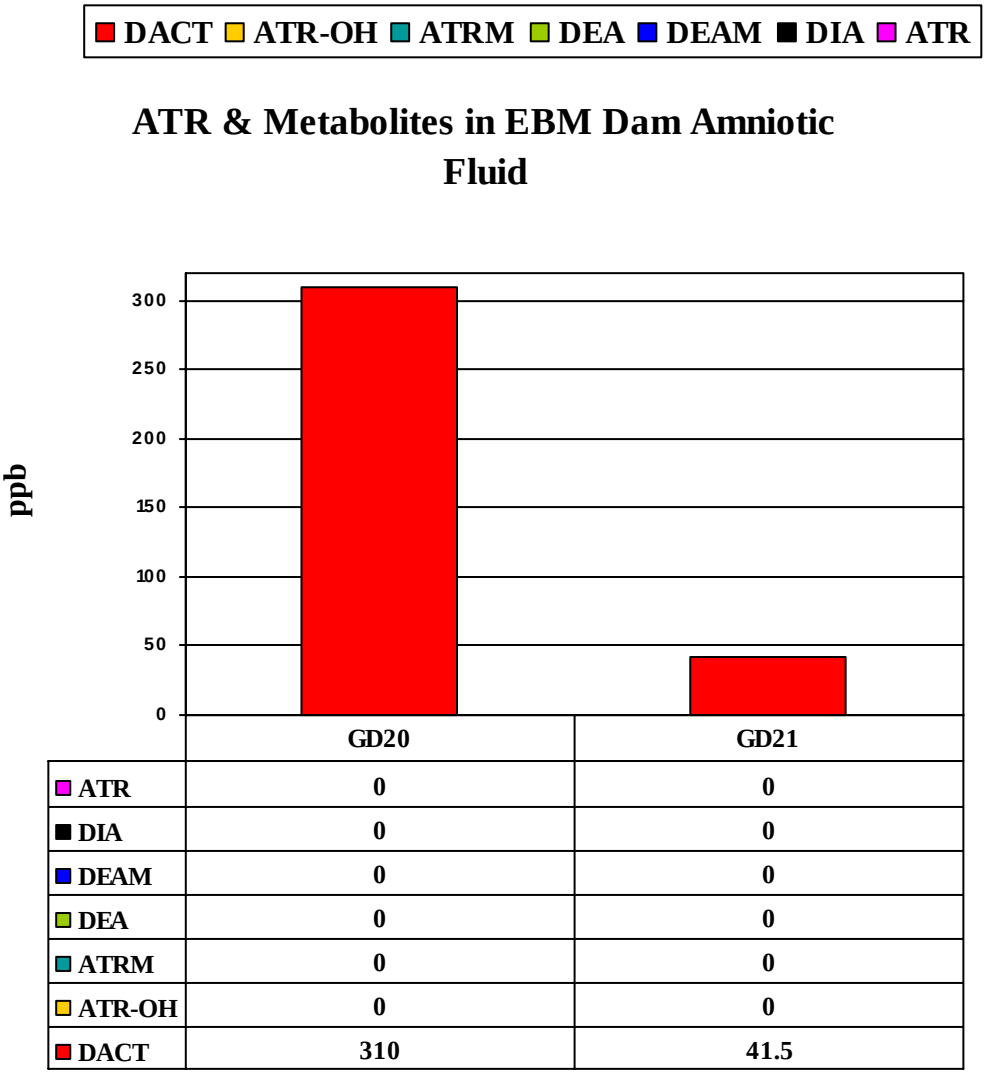
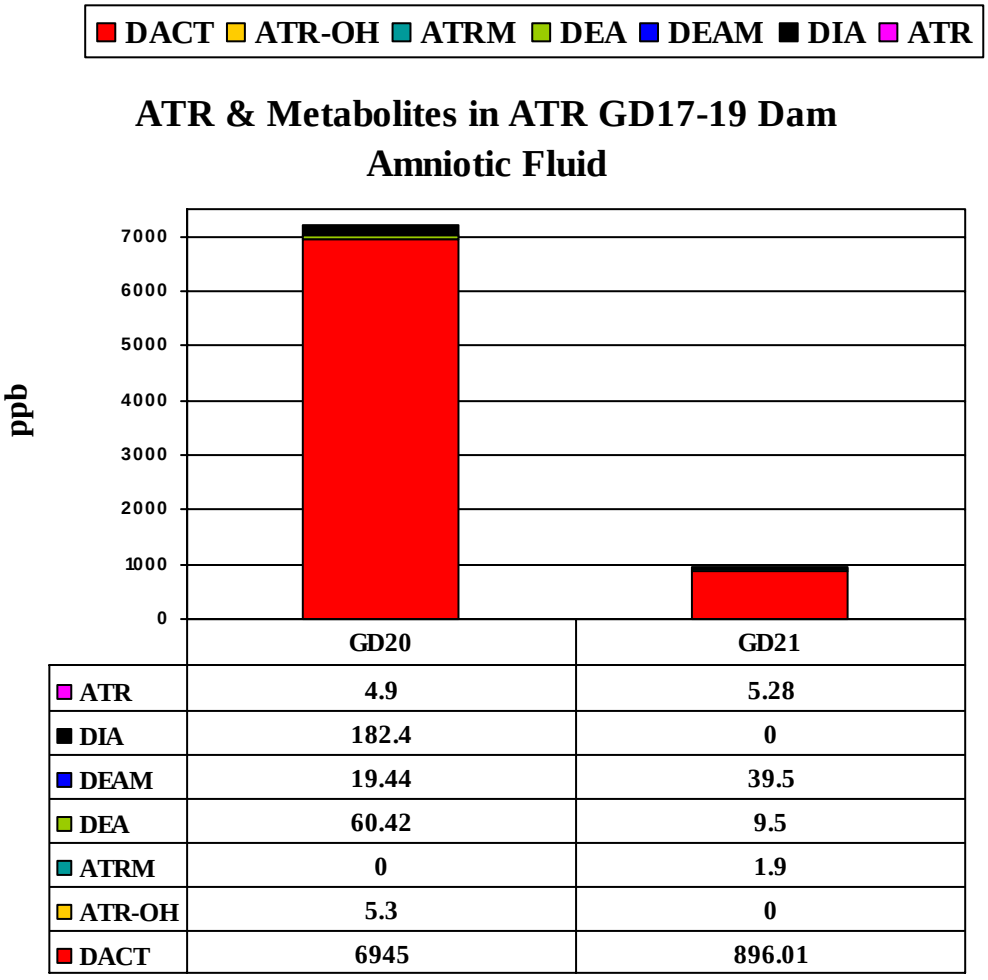
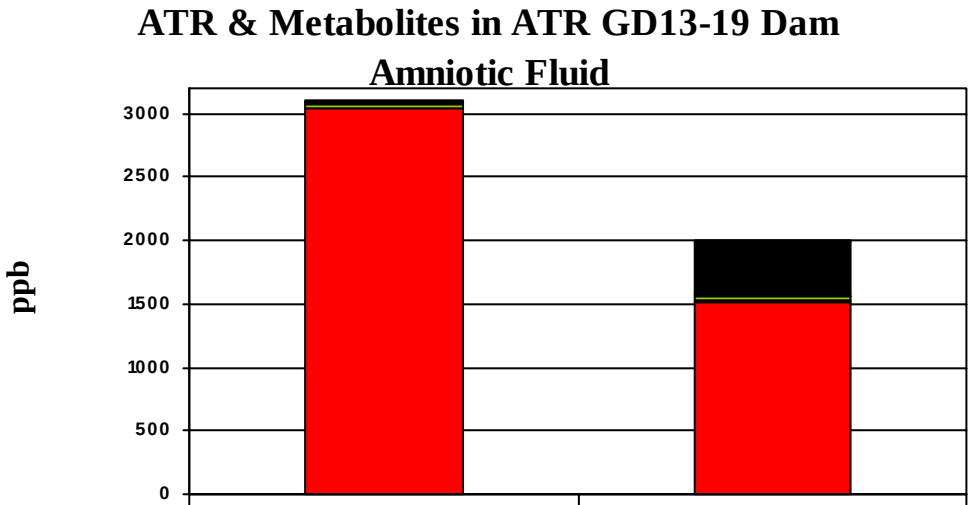


Figure 8.5. Levels of ATR and metabolites in Environmentaly Based Mixture (8.7 mg/kg) dam amniotic fluid over time. DACT was the only metabolite detected for this group of animals.



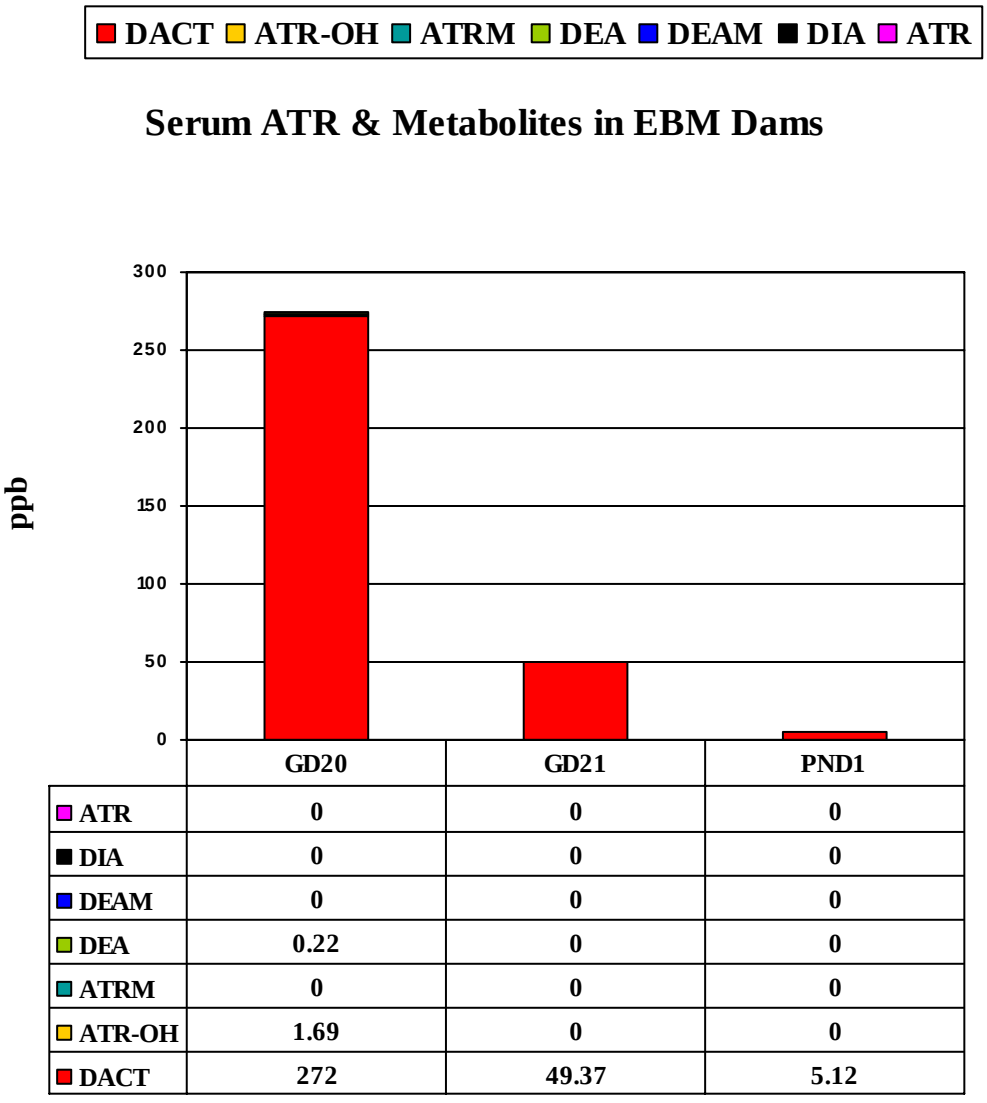
	GD20	GD21
ATR	4.9	5.28
DIA	182.4	0
DEAM	19.44	39.5
DEA	60.42	9.5
ATRM	0	1.9
ATR-OH	5.3	0
DACT	6945	896.01

Figure 8.6. Levels of ATR and metabolites in ATR GD17-19 amniotic fluid over time. DACT was the major metabolite detected but others are present in small amounts. Some parent compound was detected.



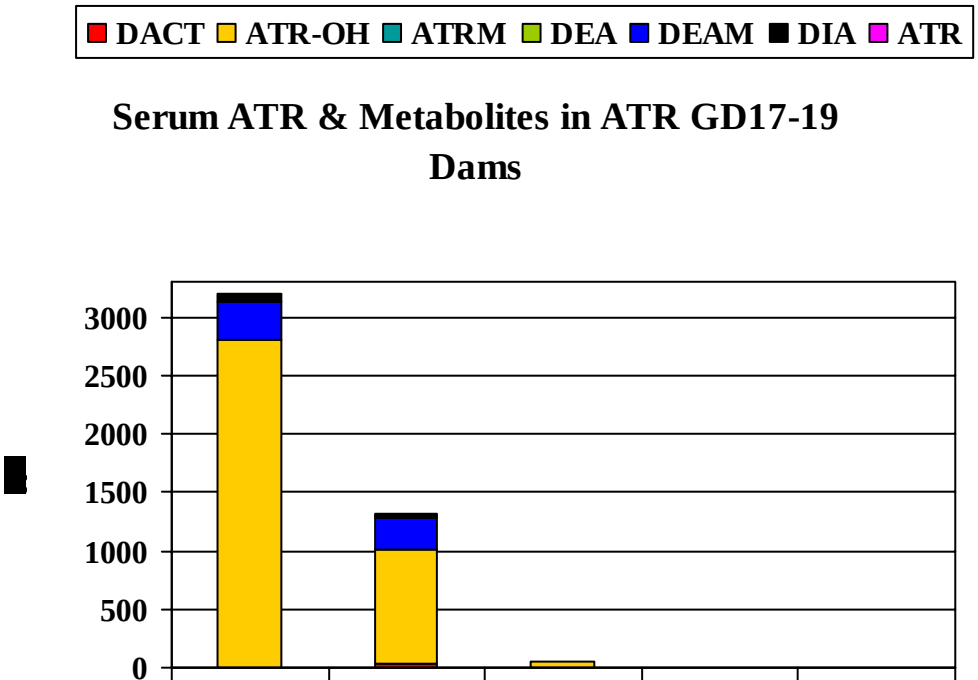
	GD20	GD21
ATR	0	25.5
DIA	19.6	402.6
DEAM	11.4	24.2
DEA	31.2	19.5
ATRM	0	5.01
ATR-OH	0	21.1
DACT	3050	1509

Figure 8.7. Levels of ATR and metabolites in ATR GD13-19 amniotic fluid over time. DACT was the major metabolite detected in these animals, but DIA was noticeably present.



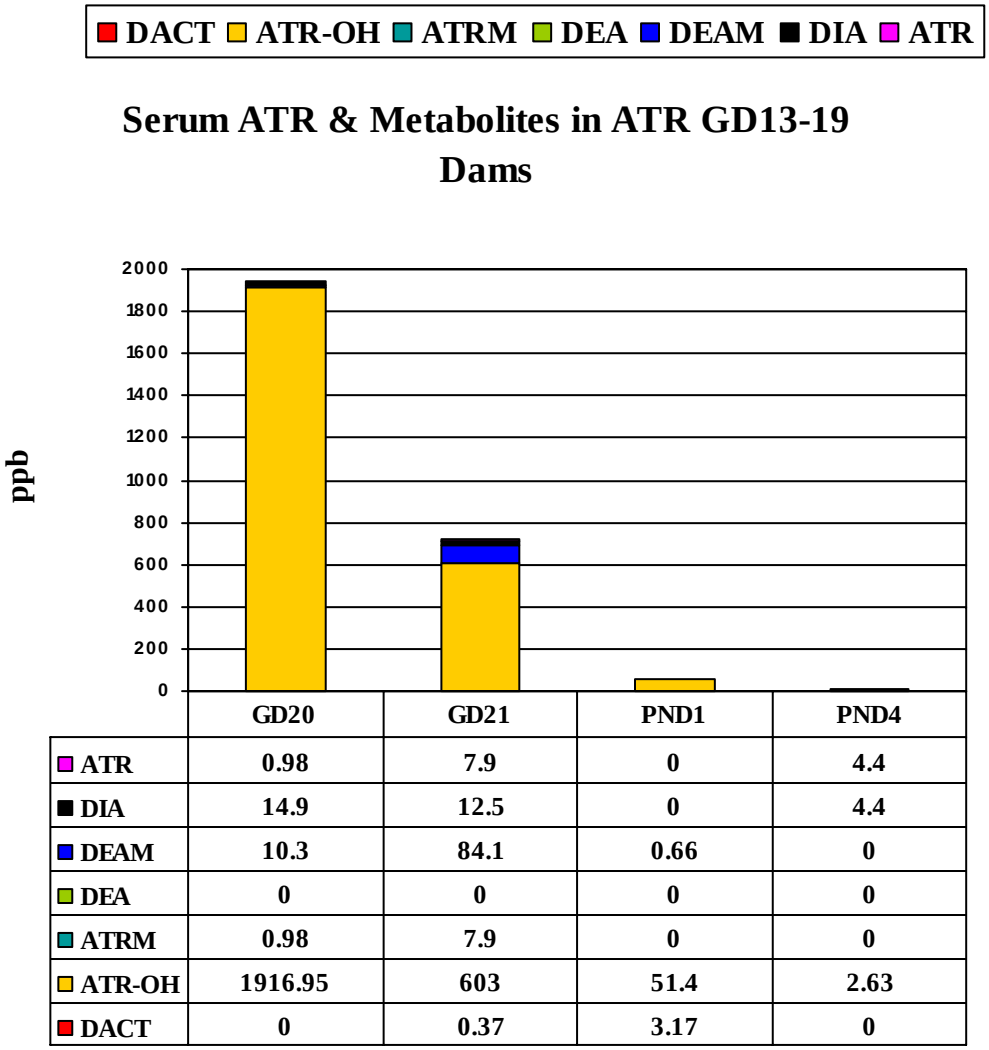
	GD20	GD21	PND1
ATR	0	0	0
DIA	0	0	0
DEAM	0	0	0
DEA	0.22	0	0
ATRM	0	0	0
ATR-OH	1.69	0	0
DACT	272	49.37	5.12

Figure 8.8. Levels of ATR and metabolites in EBM serum over time. In these animals, DACT was the major metabolite detected.



	GD20	GD21	PND1	PND4	PND11
ATR	5.7	3.7	0	0.47	0
DIA	75.1	32	0	0	0
DEAM	318	263.3	0	0.7	1.6
DEA	0.35	7.23	0	0	0
ATRM	5.7	3.7	0	0.47	0
ATR-OH	2797	965.4	57.9	4.969	6.33
DACT	1.96	35.4	0	0	0

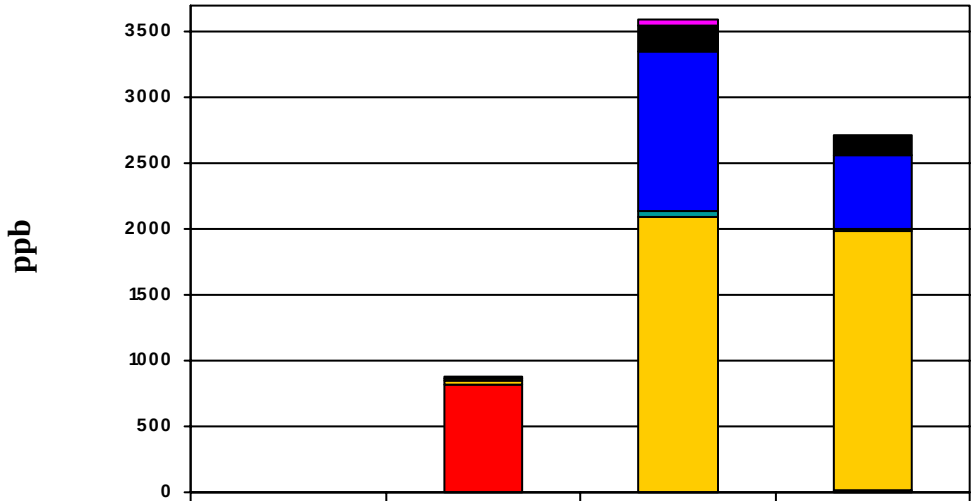
Figure 8.9. Levels of ATR and metabolites in ATR GD17-19 serum over time. In contrast to urine and amniotic fluid, ATR-OH was the major metabolite found in these animals.



	GD20	GD21	PND1	PND4
ATR	0.98	7.9	0	4.4
DIA	14.9	12.5	0	4.4
DEAM	10.3	84.1	0.66	0
DEA	0	0	0	0
ATRM	0.98	7.9	0	0
ATR-OH	1916.95	603	51.4	2.63
DACT	0	0.37	3.17	0

Figure 8.10. Levels of ATR and metabolites in ATR GD13-19 serum over time. Similar to ATR GD17-19, ATR-OH was the major metabolite detected in the serum.

Serum ATR & Metabolites in Dams 3 Hours Post Dosing on PND4



	Control	EBM	GD17-19	GD13-19
ATR	0	1.8	45.22	13.55
DIA	0	12.4	201.4	150.8
DEAM	0	0	1216	551
DEA	0	15.2	0	11.5
ATRM	0	0	45.22	13.55
ATR-OH	0	26	2090	1962
DACT	0	824.9	1.2	19.3

Figure 8.11. Levels of ATR and metabolites in serum three hours after dosing on PND4. The profiles were very similar to that detected over time. EMB animals had mostly DACT while the two ATR groups had high levels of ATR-OH and DEAM.

CHAPTER 9

CONCLUSIONS

A gestational exposure to ATR has been shown to delay mammary gland development in the offspring of Long-Evans rats. It has also been shown to alter the pregnancy-related mammary gland differentiation in the exposed dams. The effects appear to be due to small amounts of ATR as well as ATR metabolites which have been detected in the urine, amniotic fluid, and serum of the dams days after the exposure period has ended.

In these studies, we used the rat dam as the experimental unit of analysis because the dam was given the treatment dose. At this time, it is unknown how much of the actual dose and what form (parent compound or metabolites) are present and biologically active in the offspring, but the compounds do appear to reach them both transplacentally and via the milk. The exposure period ended 2 days before the dams gave birth and any exposure to ATR occurring during lactation should have been far less than the dose given to the dam as the compound is thought to be rapidly metabolized and excreted. We hypothesize that a sufficient dose of ATR can occur via the milk following a prenatal exposure to cause the effects seen in the male offspring in this study. Alternatively, ATR exposure to the dams could have inhibited milk production or quality resulting in decreased nutritive value of the milk.

Although a relatively high dose of atrazine (100 mg/kg) is used in these studies, with the exception of the low dose EBM, one must remember that each pregnant rat contains in her an average of 144 developing fetal mammary buds during the time of exposure (12 buds

per rat and an average of 12 pups/litter). Some dams contained as many as 204; possibly contributing to the litter to litter variation. This is in comparison to the 2 mammary buds developing in the average pregnant woman (lower average variation).

From the small amount of published information available on ATR disposition in the rat, it has been shown that C^{14}-labelled ATR is absorbed into plasma with a rate constant of 0.2 to 1 hr (McMullin *et al.*, 2003) and it has been reported that approximately 85% of radioactivity, administered as C^{14}-labeled atrazine, is excreted in the urine and feces of the rat in the first 72 hours after dosing (Bakke *et al.*, 1972), with the majority of that excretion (85-95%) in the first 24 hours. These excretion values are very similar to the values reported in the milk goat (Robbins *et al.*, 1968), where they further estimated that very limited amounts of labeled propazine, structurally related to ATR, were detected in milk. Furthermore, recent attempts to measure atrazine in 10 breastmilk samples found either no detectable compound or metabolite (7/10; below the limit of detection), or concentrations at (1/10) or below (2/10) the assay quantitation limit (1.0 µg/l; Balduini *et al.*, 2003).

Even though we do not yet know if ATR or its metabolites are transferred in the milk or changing the composition of the milk, we do know that gestational exposure to ATR or a metabolite mixture changes the dam's mammary gland development. We know that ATR and its metabolites are present in the dam as late as PND11 as evidenced by urine metabolite levels and PND4 in serum. We also have preliminary evidence that metabolites are detected in mammary gland tissue from pregnant and lactating ATR treated dams suggesting that ATR or at least its metabolites are transported into the tissue or the milk during gestational exposure.

You and colleagues (1999) demonstrated that 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE) was stored in the mammary glands following *in utero* exposure and released during nursing. When litters were cross-fostered, the lactational DDE exposure to the pups proved greater than that of the *in utero* exposure. There are many pathways through which endogenous and exogenous substances enter milk including membrane or glucose transport, through mammary epithelial cell lipid secretion, or active transport mechanisms (McManaman and Neville, 2003). You and colleagues (1999) suggested DDE, a lipophilic compound, associated with fatty acids or lipoproteins in mammary tissue used for milk production. It is possible that ATR acts in a similar manner, being stored in the dam's body and released with milk production. We have observed delayed mammary gland development in offspring only exposed *in utero* or only through nursing in the exposure parameters studies as well as in the cross-fostering within each critical period studies.

We suggest that ATR exposure to the pregnant dam may alter her milk composition. It has been shown that 100 mg/kg/d ATR inhibits suckling-induced prolactin surges in Wistar rats (Stoker *et al.*, 1999). From the number of papers evaluating hormonal control of development of the mouse mammary gland (reviewed in Rosen *et al*., 1994 and Silberstein, 2001), it is known that many hormones and growth factors contribute to the correct development of the rodent mammary gland. Disruption of one or several of those factors (many of which are milk-borne) may contribute to the delay in development seen in these studies. Additionally, it is possible that hormone production other than those we evaluated contribute to our findings. However, we do not suspect that altered maternal behavior or a lack of milk production by the dam is the moderator of effect since a relatively small change in body weight was seen in the offspring due to treatment. Further, we see these as early

effects, interrupting local growth factor-regulated development of the mammary bud, or early neonatal outgrowth of the gland as the effects of exposure are apparent as early as PND 4.

It did not appear that the mammary gland delays observed in the offspring were the result of changes in puberty in the animals. Early mammary gland development is controlled by many hormones and local growth factors. As stated earlier, estrogen, progesterone, and prolactin are important to the proper growth and development of the mammary gland. Growth hormone acts on stromal cells to stimulate end bud formation and mammary epithelial proliferation. EGF and insulin-like growth factor-I and II play essential roles in mammary gland development. Members of the transforming growth factor-β family have been found to be involved in both ductal and lobuloalveolar development (Silberstein, 2001). Even though we did see a delay in VO and mammary development, our statistical analyses did not show an interaction between these endpoints, suggesting that the delay in VO was not associated with the developmental delays seen in the mammary gland. We propose, rather, that an early imprinting effect, either via transplacental exposure to atrazine or early lactational exposures may be the cause of the delays seen in peripubertal mammary development.

From puberty to adulthood, the rodent mammary gland undergoes allometric growth, characterized by the mammary gland growing nearly 4 times faster than body surface area. This growth is dependent on ovarian hormone and local factors (Daniel and Silberstein, 1987). Sanderson *et al.* (2000) reported the induction of aromatase by ATR in a human cell line and Yue *et al.* (2001) reported the moderate down-regulation of aromatase activity in breast cancer cells by estrogen suggesting that estrogen plays a role in regulating aromatase in the breast. Oka *et al.* (1991) suggests that EGFR numbers in the mammary gland are high

during proliferative phases and low when the gland is in its resting state. Growth factors and enzymes that bind EGFR, in addition to endocrine factors that regulate its expression, may be important in controlling mammary gland growth and development via stromal-epithelial interactions. Wiesen *et al.* (1999) reported that EGFR is essential to induce estrogen-dependent ductal growth and branching morphogenesis. From this, they suggest that EGFR-mediated signaling, via estrogen action, is required for stimulation of epithelial growth and development. During this period of time, PND33, we found that animals within the ATR exposure parameters had less aromatase and EGFR RNA expressed in their mammary glands. Our data suggest that ATR exposure can decrease aromatase and EGFR expression in the mammary gland, but the mechanism by which these events occur is unknown.

The mode of action of atrazine for the developmental delays in the rat mammary gland is unknown. There were no changes in puberty or hormones that could be associated with delayed mammary gland development in our study. However, we know that body weight is not the answer. Only the offspring from dams dosed GD13-19 in the critical period study exhibited delayed VO, indicating again that VO is not altered in a time/dose paradigm similar to ATR-induced mammary gland development delays. Delayed VO seems to require longer ATR exposures than does the notable mammary changes. This situation is reminiscent of pubertal changes in U.S. girls, where the timing of breast development and menses do not necessarily go hand-in-hand. Data from over 17,000 girls (Herman-Giddens *et al.*, 1997) and the Third National Health and Nutrition Examination Survey (Sun *et al.*, 2002) demonstrated that breast and pubic hair development are beginning earlier in both African-American and Caucasian populations and taking longer to conclude. However, menses occurs at an age similar in these populations as that seen 30 years ago (Chumlea *et*

al., 2003). Many theories exist on the effect of the environment on these changes in sexual maturity. In our critical period study, the hormone concentrations measured from serum did not differ consistently among the groups, even when considered by stage of the estrous cycle. Statistical differences in hormone concentrations at PND33 did not persist to PND67 and were not outside the biologically normal value ranges. This further indicates that serum hormone levels are not a direct causal factor in delaying mammary gland development following ATR exposure. Once again, we go back to the possibility that the effect of ATR is an early imprinting effect and that the programmed growth pattern of the mammary epithelium was altered by the prenatal or early postnatal exposures.

The effect of ATR on puberty could be explained by many different theories including hormonal changes or early postnatal brain development (specifically in the median eminence), critical to regulating the timing of vaginal opening, were altered by ATR and led to delays. Atrazine may affect the enzymes or local growth factors in the median eminence that regulate LHRH release (Ojeda *et al.*, 2000), the levels of ghrelin (a negative regulator of LH secretion and a marker of energy insufficiency; Fernandez-Fernandez *et al.*, 2005), or possibly the circulating levels of leptin in the offspring were altered due to decreased appetite in the dams during the perinatal period. Plasma leptin levels correlate with pubertal timing in rats (Leonhardt *et al.*, 2003). Altered maternal prolactin following ATR-exposure (Cooper et al., 2000) may influence these or other critical elements of pubertal timing in the offspring. Although we have no data to support any of these potential mechanisms of action for perinatal ATR exposure on pubertal delays, they are all consistent with known ATR modes of action (CNS and appetite-related effects).

Either low levels of ATR or its metabolites are readily present in the milk of these animals or the milk quality of ATR-exposed dams is sufficiently compromised to cause the puberty delays. From the lactational challenge studies (Appendix B) we know that the ATR-exposed pups are getting similar amounts of milk. Atrazine metabolism follows first-order kinetics, and is reported to be rapidly eliminated from the body of adult non-lactating rats. For example, in adult female Sprague-Dawley rats given 100 mg/kg radio-labeled ATR (orally), 100% of the compound was recovered in 3 days (Atrazine Toxicology, 2002). Whether or not rapid elimination takes place in the pregnant and lactating dam is still in question. The mode and mechanism of action of atrazine on pubertal timing is unknown and deserves further investigation, focusing on exposure during the early postnatal period.

In conclusion, my studies on Long Evans rats exposed to ATR prenatally have shown that brief *in utero* exposures to ATR, parent compound and/or low dose mixture, delays mammary gland development in the female offspring as early as PND4. The mammary gland effects are not associated with body weight changes, puberty or serum hormone concentrations later in life. I have shown that a critical window of susceptibility (GD17-19) exists during fetal mammary gland development. During this time, the fetus of ATR treated dams is exposed to DACT and other metabolites in the amniotic fluid. When the gland is exposed to ATR during this time, it leads to delayed mammary gland development that persists into adulthood. These animals then have difficulty providing enough milk for their offspring. I have also shown that ATR and/or metabolite associated changes (ATR/metabolites in milk or milk composition changes) in the dam dosed during late gestation (after GD17) can be transferred to naïve offspring and yield similar delayed mammary gland development as those seen in gestationally exposed offspring. These studies

have shown that ATR accelerates pregnancy-related dam mammary gland development, and its metabolites can be detected in the dam as late as 13 days post dosing suggesting that metabolism of ATR is different in the pregnant and lactating rat than in the virgin rat. From these data, it can be suggested that regulatory attention be given to ATR metabolites, some of which are known to be breakdown products of other chlorinated triazine pesticides.

These studies have aided the Centers for Disease Control in achieving excellent quality standards and protocols for measuring ATR and metabolites in the human population. As well, they give insight into possible wildlife implications (low dose exposure) especially with respect to lactation and adverse effects on future generations.

APPENDIX A

ATRAZINE AND METABOLITE CONCENTRATIONS

Millimolar Concentration of Atrazine Metabolites Detected in EBM GD13-19 Dam Urine

	GD19	GD19U	GD20	GD21	PND4	PND11
DACT	36	27	24	10	70	8.6
ATR-OH	38	15	2.5	10	11	0.70
ATRM	0.27	0.06	0.17	0.10	0.004	0
DEA	0.71	0.04	0.31	0.13	0.05	0
DEAM	7.7	2.3	0.85	5.5	0.71	0
DIA	1.0	0.1	1.3	0.50	0.24	0
ATR	0.84	0	0.004	0	0	0

Value of 0 indicates concentration below the limits of detection.

Millimolar Concentration of Atrazine Metabolites Detected in GD ATR17-19 Dam Urine

	GD19	GD19U	GD20	GD21	PND4	PND11
DACT	73	26	124	34	1.7	173
ATR-OH	30	13	6.3	0.43	0.32	4.5
ATRM	2.0	0.94	1.0	0.032	0.22	0.57
DEA	4.4	2.6	1.1	0.12	0.19	0.15
DEAM	27	38	13	0.22	1.5	7.7
DIA	12	6.4	3.5	0.10	0.64	1.9
ATR	3.2	1.4	0.62	0.009	0.41	1.1

Value of 0 indicates concentration below the limits of detection.

Millimolar Concentration of Atrazine Metabolites Detected in GD ATR13-19 Dam Urine							
	GD19	GD19U	GD20	GD21	PND1	PND4	PND11
DACT	157	52	134	48	41	150	13
ATR-OH	9.8	4.0	0.73	9.7	2.6	6.2	2.1
ATRM	1.4	1.4	0.18	0.14	1.0	1.3	0.28
DEA	5.5	2.8	0.80	0.90	5.6	3.4	1.0
DEAM	27.4	22	2.8	1.6	18	17	4.3
DIA	9.5	8.0	0.87	1.9	14	7.2	1.1
ATR	2.2	1.4	0.15	0.083	3.6	2.1	0.44
Value of 0 indicates concentration below the limits of detection.							

Millimolar Concentration of Atrazine Metabolites Detected in Dam Urine on PND4 3 Hours Post-dosing				
	Control	EBM GD13-19	GD17-19	GD13-19
DACT	0	23	71	294
ATR-OH	0	18	3.7	11
ATRM	0	0.15	1.1	2.2
DEA	0	0.26	2.1	7,1
DEAM	0	1.6	8.3	15
DIA	0	0.42	6.1	9.1
ATR	0	0.007	2.7	3.3
Value of 0 indicates concentration below the limits of detection.				

Millimolar Concentration of Atrazine Metabolites Detected in EBM GD13-19 Dam Amniotic Fluid		
	GD20	GD21
DACT	2.1	0.28
ATR-OH	0	0
ATRM	0	0
DEA	0	0
DEAM	0	0
DIA	0	0
ATR	0	0
Value of 0 indicates concentration below the limits of detection.		

Millimolar Concentration of Atrazine Metabolites Detected in ATR GD17-19 Dam Amniotic Fluid		
	GD20	GD21
DACT	48	6.2
ATR-OH	0.026	0
ATRM	0	0.005
DEA	0.32	0.051
DEAM	0.065	0.13
DIA	1.0	0
ATR	0.023	0.024
Value of 0 indicates concentration below the limits of detection.		

Millimolar Concentration of Atrazine Metabolites Detected in ATR GD13-19 Dam Amniotic Fluid		
	GD20	GD21
DACT	21	10
ATR-OH	0	0.11
ATRM	0	0.015
DEA	0.16	0.10
DEAM	0.038	0.081
DIA	0.11	2.3
ATR	0	0.11
Value of 0 indicates concentration below the limits of detection.		

Millimolar Concentration of Atrazine Metabolites Detected in EBM GD13-19 Dam Serum			
	GD20	**GD21**	**PND1**
DACT	**1.9**	**0.34**	**0.035**
ATR-OH	**0.008**	**0**	**0**
ATRM	**0**	**0**	**0**
DEA	**0.001**	**0**	**0**
DEAM	**0**	**0**	**0**
DIA	**0**	**0**	**0**
ATR	**0**	**0**	**0**
Value of 0 indicates concentration below the limits of detection.			

Millimolar Concentration of Atrazine Metabolites Detected in ATR GD17-19 Dam Serum					
	GD20	**GD21**	**PND1**	**PND4**	**PND11**
DACT	**0.013**	**0.24**	**0**	**0**	**0**
ATR-OH	**14**	**4.9**	**0.29**	**0.025**	**0.032**
ATRM	**0.017**	**0.011**	**0**	**0.001**	**0**
DEA	**0.002**	**0.038**	**0**	**0**	**0**
DEAM	**1.2**	**0.88**	**0**	**0.002**	**0.005**
DIA	**0.43**	**0.18**	**0**	**0**	**0**
ATR	**0.026**	**0.017**	**0**	**0.002**	**0**
Value of 0 indicates concentration below the limits of detection.					

Millimolar Concentration of Atrazine Metabolites Detected in ATR GD13-19 Dam Serum				
	GD20	**GD21**	**PND1**	**PND4**
DACT	**0**	**0.002**	**0.022**	**0**
ATR-OH	**9.7**	**3.0**	**0.26**	**0.013**
ATRM	**0.003**	**0.023**	**0**	**0**
DEA	**0**	**0**	**0**	**0**
DEAM	**0.034**	**0.28**	**0.002**	**0**
DIA	**0.086**	**0.072**	**0**	**0.025**
ATR	**0.004**	**0.037**	**0**	**0.020**
Value of 0 indicates concentration below the limits of detection.				

Millimolar Concentration of Atrazine Metabolites Detected in Dam Serum on PND4 3 Hours Post-dosing				
	Control	**EBM GD13-19**	**GD17-19**	**GD13-19**
DACT	**0**	**5.7**	**0.008**	**0.13**
ATR-OH	**0**	**0.12**	**10.6**	**9.9**
ATRM	**0**	**0**	**0.13**	**0.040**
DEA	**0**	**0.081**	**0**	**0.061**
DEAM	**0**	**0**	**4.1**	**1.8**
DIA	**0**	**0.071**	**1.2**	**0.87**
ATR	**0**	**0.08**	**0.21**	**0.063**
Value of 0 indicates concentration below the limits of detection.				

APPENDIX B

ATRAZINE AND MATERNAL BEHAVIOR

During the dosing period, dams are monitored closely to determine if treatment is having an adverse effect on the dam. Dams are monitored following dosing to determine treatment effects on parturition and maternal behavior. We observed ATR dams and their offspring, and hypothesized that ATR may alter maternal behavior leading to reduced weight gain of their offspring.

Methods

These studies were performed within previous experiments.

Animals-Time-pregnant Long Evans rats (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). The animals were housed in an AAALAC accredited facility, one per cage and given food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. They were maintained in a room with a 14:10 hour light cycle, 20-24°C and relative humidity of 40-50%. Animal protocols were reviewed and approved by the National Health and Environmental Effects Research Laboratory, Institutional Animal Care and Use Committee.

Dosing Solution and Procedures- ATR (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. The environmentally based mixture (EBM) was prepared as a suspension in 1.0% methyl cellulose (Sigma) in distilled water. The EBM is a mixture of atrazine and its metabolites developed from the data accumulated from the North Carolina State Extension office and based on the highest measured levels of atrazine metabolites. It consists of atrazine (25%), 2-chloro-4, 6-diamino-s-chlorotriazine (DACT 35%), hydroxyatrazine (HA 20%), 2-chloro-4-amino-6-(isopropylamino)-s-triazine (DEA 15%), and 2-chloro-4-amino-6-(ethylamino)-s-triazine (DIA 5%). Time pregnant rats were treated in the morning with 0 (vehicle), EBM (total concentration equal to 8.7 mg/kg), or 100 mg atrazine/kg BW by oral gavage in 5 ml/kg dosing volume. This ATR reference dose (100 mg/kg/d) was chosen due to consistent reproductive endpoint effects observed in previous studies (Laws *et al.,* 2000; Rayner *et al.*, 2004; Stoker *et al.,* 1999).

Experimental Design- Ninety six pregnant LE dams were treated with vehicle (control), 8.7 mg/kg EBM, or 100 mg/kg ATR daily (N>9 dams/ exposure period/treatment). Control and EBM dams were dosed gestational days (GD) 13-19 or GD15-19, and dams receiving ATR were dosed GD17-19, GD15-19, or GD13-19. On PND4 remaining litters were weighed and randomly equalized to 10 pups. On PND11, dams and pups were lactationally challenged.

Lactational Challenge- On PND10 dams and pups were moved to a quiet holding room. On PND11, dams were removed, weighed, and placed in clean individual cages with food and water *ad libitum*. The bellies of the pups were rubbed to encourage urination, and then pups were individually weighed and placed back in their own nest. Three hours later, dams were placed back with their pups. The amount of time it took for dams to nest on their young was recorded, and dams were allowed to suckle their pups for 30 minutes after which dams were removed and decapitated. Litters were reweighed immediately and euthanized.

Mammary Gland Scoring- Dam mammary glands were subjectively scored. Dam developmental scores were based on the percentage of the gland filled with lobuloalveolar units, the size of the units within each structure, and the amount of distention (by lobuloalveolar units) within the gland. When the lobuloalveolar units were not empty, a score was given to the amount of secretory product present and the location of the product.

Statistical Analysis- Group means and dam means (pups/dam; litter as unit) were calculated for body weights and time-to-nest and were evaluated for treatment effects within each age group by one-way analysis of variance (ANOVA, Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC).

Results

Maternal Behavior- When dams were placed back into their cages after a three hour separation, they were watched and timed closely to determine the amount of time to settle over their litters in the arched-back crouched position and allow the pups to nurse. The time-to-nest was not found to be different among the different treatment groups. These data demonstrated that ATR does not change this aspect of maternal behavior.

Although litters were culled to 10 pups on PND4, the average number of pups remaining on PND11 was 9.5 pups per litter per treatment group. Litter weights prior to lactational challenge were not found to be statistically different among groups. Following nursing, litters were weighed again to determine weight gain. No statistical differences were found in litters weights or weight gain post nursing. Weigh gain per pup were similar for all groups. These data suggest that dam milk quantity is not altered in ATR treated dams.

Pregnancy related dam mammary gland development- Dam mammary glands were examined for changes related to treatment following the lactational challenge. They were scored for different developmental components as well as for content in the lobuloalveolar units. No differences were found among the groups for development or content. This data suggest that there are no dam mammary gland differences at PND11.

APPENDIX C

ATRAZINE-INDUCED REPRODUCTIVE TRACT ALTERATIONS AFTER TRANSPLACENTAL AND/OR LACTATIONAL EXPOSURE IN MALE LONG-EVANS RATS

These studies examine the effects observed after short-term prenatal exposure to ATR in Long Evans rats on pubertal timing, reproductive tissues, and hormone levels in the male offspring. The effects on their female siblings were reported elsewhere. Exposure parameters were designed to determine if exposure-induced changes were the result of transplacental or lactation exposure to ATR, or both. We report male reproductive tissue effects following gestational exposure. We have also evaluated the effects on the prostate via myeloperoxidase assays and pathological examination at both postnatal days 120 and 220. Our results suggest that late gestational exposure to atrazine and/or the resulting lactational effects of the prenatal exposure can influence prostate weight, hormone levels, and inflammation, long after the exposure has occurred, presumably due to its effects on developing tissues at the time of the exposure.

Methods

Animals- Time-pregnant Long-Evans rats (LE) (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). Dams were housed one per cage in an AAALAC-International accredited facility and provided food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. The rats were maintained in a room with a 14:10 hour light cycle, 20-24°C and relative humidity of approximately 50%. Animal protocols were reviewed and approved by the National Health and Environmental Effects Research Laboratory (NHEERL), Institutional Animal Care and Use Committee. Animals tested negative for infectious diseases prior to beginning the study.

Dosing Solution- Atrazine (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. Timed-pregnant rats were treated with vehicle or 100 mg ATR/kg BW by oral gavage in 5 ml/kg dosing volume. The dose of ATR was chosen based on consistent results on reproductive tissues in previous studies (Laws *et al.,* 2000; Rayner *et al.*, 2004; Stoker *et al.*, 1999a).

Experimental Design- The general study design has previously been reported (Rayner et al., 2004). Briefly, 40 pregnant LE dams were treated on gestational days (GD)15-19 (time period during which reproductive tissues are developing) with vehicle, C, or 100 mg/kg ATR/BW. On PND1 (day of birth), litters were weighed and half litters were cross-fostered with half of another litter from a vehicle or ATR treated dam, creating four exposure parameters: dam born to or gestational exposure only (ATR-C), milk source or lactational exposure only (C-ATR), exposure both pre- and postnatal (ATR-ATR), or neither (C-C). Pups were individually identified. Litters were weighed and randomly equalized to 10 pups

on PND4. At weaning, PND22, animals were weighed and separated into unisex sibling groups, with two male rats per cage. Males were singly housed if aggressive behavior was observed within a cage. Males were euthanized and necropsied on PND120 or 220.

Preputial Separation- Beginning on PND 37, male offspring (N>18 males/group) were evaluated for puberty. The separation of the foreskin of the penis from the glans (preputial separation, PPS) was used to determine if an animal had gone through puberty (Korenbrot *et al., 1977*). The day of complete PPS and body weight on that day were recorded.

Necropsy- Necropsies were performed following an overnight and continued stay in a quiet holding area, and by using decapicones for animal transfer to reduce stress. Males were euthanized on either PND120 (N=10/group) or 220 (N>8/group). Following decapitation, trunk blood was collected into serum separator tubes and centrifuged for 30 minutes at 3000 rpm (4ºC) to obtain serum for hormone assays. The pituitary gland, testes, lateral and ventral prostates, and seminal vesicles of each animal were removed and individually weighed. Gross lesions were recorded. The right lateral and ventral prostates were fixed in 10% neutral buffered formalin for routine histology. Pituitary glands and left lateral prostates were placed on dry ice, frozen, and stored at -80°C.

Radioimmunoassays- Sera obtained from male offspring PNDs 120 and 220 were analyzed for prolactin levels by radioimmunoassay using materials supplied by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases. The prolactin assay was run as previously described in detail by Stoker *et al.,* (2000). Total serum testosterone and androstenedione were measured using Coat-a-Count Radioimmunoassay Kits obtained from Diagnostic Products Corporation (Los Angeles, CA). Serum estrone was measured using the DSL 8700 Estrone Radioimmunoassay kit from Diagnostic Systems Laboratories, Inc.

(Webster, TX). All assays were run in duplicate following manufacturer's directions. Inter-assay coefficients of variation for the prolactin, testosterone, adrostenedione, and estrone assays were 5.2%, 4.7%, 4.4%, and 4.9% respectively.

Myeloperoxidase Assay- The left lateral prostates collected on PNDs120 and 220 were analyzed for myeloperoxidase (MPO) activity. Tissues were homogenized and prepared as described in detail by Stoker *et al.*, (1999a) for myeloperoxidase assays. The supernatant (250 µl) of the tissue homogenate was assayed using the APLCO Myleperoxidase ELISA kit (American Laboratory Products Company, Windham, NH) following the manufacturer's directions. Duplicate samples were loaded onto a 96-well plate coated with anti-MPO antibody. A peroxidase-conjugated polyclonal anti-MPO antibody was added followed by tetramethylbenzidine and a stop solution. The plate was read on a Spectra Max Plus Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) at an excitation wavelength of 450 nm. The standard curve used in the assays had correlation coefficients of R=0.99 (PND120) and R=1.0 (PND220). The data was used to calculate the MPO concentration in each sample according to the manufacturer's directions.

Histology and Pathology- The right lateral and ventral prostates which were fixed in 10% neutral buffered formalin, and were processed by routine methods to paraffin block. Five slides were prepared from each gland; 5 µm sections were taken following step-sectioning through the gland (skipping 20 µm segments). They were stained with hemotoxylin and eosin (H&E) for microscopic examination. All 5 slides from a single prostatic lobe were examined without knowledge of treatment and graded based on the distribution (% of gland affected) and severity (magnitude of cellular infiltrate) of the inflammation. Distribution scores were as follows: 0- none, 1 - 10% or less of gland affected, 2 – approximately 25%, 3 –

approximately 50%, 4 – 75% or greater of the gland affected. For severity of inflammation in organ: 0 – no infiltration of inflammatory cells, 1 - minimal interstitial infiltration of inflammatory cells, 2 - moderate interstitial inflammation with or without focally marked accumulations of perivascular collections of inflammatory cells, 3 - early micro-abscess formation, mild numbers of inflammatory cells in acinus with some degradation of colloid, 4 – micro-abscess, 5- micro-abscess with moderate to marked interstitial inflammation. If two or more slides per gland were found to have inflammation a score was given and averaged according to the most severe lesion per slide. No score was given if a lesion was found on only one slide. In addition, the cellular component of the inflammatory process was identified. Lymphocytes were most commonly present. If neutrophils made up more than 10% of the inflammatory cell population then both the diagnosis of lymphocytes and neutrophils were made. If neutrophils were more than 90% of the cell population then only the diagnosis of neutrophils was made. The prostates were also examined for the presence of edema which was characterized by expansion of the interstitial spaces between the glandular acini with eosinophilic proteinaceous material present.

Statistical Analysis- Dam means (vs. litter means, because half litters were crossed) were calculated for PPS, as well as male offspring body and tissue weights. Data were evaluated for age and treatment effects by analysis of variance (ANOVA) using least-squares regression model (LSM) (Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC). Means were evaluated and treatment groups compared to each other. Means and adjusted means relative to body weight were calculated for organ weights. These means were compared with control means and among treatment groups. Body weights, serum hormone concentrations, and MPO concentrations were analyzed by one-way ANOVA. Visual

evidence of prostate abnormalities was analyzed in a contingency table using Fisher's exact test in Graphpad Instat (Graphpad Software, San Diego, CA). Prostate pathology distribution and severity scores were analyzed for treatment and age effect by t-test and Dunnett's multiple comparison in JMP (SAS). Significant treatment effects were indicated when $p<0.05$.

Results

Growth and Puberty- The male offspring in the ATR-ATR group were significantly smaller ($p<0.001$) at PND4 when compared to offspring in the C-C and C-ATR groups (8.6±0.1g ATR-ATR vs. 9.2±0.1g C-C, 9.0±0.2g ATR-C, and 9.2±0.1g C-ATR). Although statistically significant, the offspring were only found to have a 6.5% reduction in weight, and this is within normal biological size variation. This small decrement in weight was also reported in ATR-ATR exposed female siblings (Chapter 4), but is not due to lack of weight gain *in utero* (Chapter 5). At weaning, PND22, the treatment groups were all similar in body weight (56.9±1.0g C-C, 54.6±1.1g ATR-C, 54.6±1.0 g C-ATR, and 56.0±0.9g ATR-ATR). Body weights in the C-ATR males were reduced 8.4%, but other males were not different at PND120 (Table 1). Animals from the ATR-C group were significantly smaller ($p<0.05$) than animals in the control and C-ATR groups at PND220 (Table 1).

Male offspring were evaluated for preputial separation (PPS) as a physical indication of puberty. Control males displayed PPS on PND 41.3±0.3 (Fig. 1A). A significant 2 ½ day delay was seen only in males in the ATR-ATR group (43.7±0.5, $p<0.01$). This delay was also significant when compared to males in the remaining groups. Body weights at time of PPS were decreased in the ATR-ATR animals ($p<0.01$ vs. C-C, Fig. 1B). While a significant reduction in body weight was not seen in the remaining ATR groups, there was a trend for body weight reduction (3-7%) at time of puberty, in addition to a 1.5 day ($p=0.07$) delay in mean day of PPS in the C-ATR group.

Reproductive Tissue Weights- Reproductive tissues and pituitaries were removed from male rats on PND 120 or 220. There were no significant weight differences among the groups for pituitary glands in rats euthanized at PND120 (Table 1). The pituitary glands

from the prenatally ATR exposed males weighed significantly more than those taken from controls at PND220, regardless of when the exposure occurred. However, when controlling for body weight of the animals in the distinct treatment groups, pituitary gland weights of animals in the ATR-C and ATR-ATR treatment groups were significantly increased compared to control animals ($p<0.03$).

Significant changes in weight were not detected or present for other male reproductive tissues from the various ATR exposures (Table 1, whether or not body weight was a covariate), there was a consistently significant difference in lateral prostate weights at both time points. At PND120, the lateral prostates of the ATR-ATR offspring group weighed significantly more ($p<0.01$) than those in any other group (Fig. 2). When lateral prostate weights were compared using body weight as a covariate, they were still found to be significantly different ($p<0.01$) when compared to all other groups. The average ATR-ATR lateral prostate weight was 49% higher than control. At PND220, lateral prostate weights were significantly increased in animals within all ATR exposure groups compared to control animals, 45-110%. When adjusted for BW (statistical differences shown in Fig. 2), animals in ATR-C and ATR-ATR groups had significantly increased lateral prostate weights ($p<0.01$). While lateral prostate weights from the ATR-ATR males were significantly different from all other groups ($p<0.03$), weights from males in the ATR-C group were considered different from C-C and ATR-ATR ($p<0.03$). The greater average body weight in the PND220 C-ATR group reduced the apparent significance of the lateral prostate weight effect.

At necropsy, seven of ten offspring in the ATR-ATR group had macroscopic abnormalities characterized by nodular pale white foci present on the outer surface of the

lateral prostates at PND120 (three on right lateral, four on left lateral prostate, Table 2) which was statistically different from C-C and ATR-C group as both lacked macroscopic alterations. The C-ATR group had four of ten males with macroscopic prostate alterations. All were found on the right lateral prostate. At PND220, visible abnormalities were present in all ATR exposed groups. The majority were found on the right lateral prostate. As shown, ATR-C males had 22% and ATR-C had 40% displaying macroscopic nodules. ATR-ATR had 56% with alterations which was significantly different from C-C group which had none visible foci.

Histopathology- At PND120, the distribution of inflammation in the lateral prostates of males from the C-ATR group was significantly increased compared to control males ($p<0.05$, Fig 3, Table 3) which was localized to focal areas of the tissue. Affected glands had high multiple acini affected with inflammation present in the interstitial space and the acinus, and micro-abscesses (Fig. 3). The C-ATR and ATR-ATR glands had neutrophils as the predominant inflammatory cell. They were more prominent in the ventral prostate (Figure 4) in all dose groups, and associated with a higher incidence of inflammation in the ventral prostate sections at PND120 (Table 3). The inflammation in the ventral prostate was not dose related and presented a wider tissue distribution than the lateral prostates. The severity of inflammation in the ventral prostate was not affected by ATR exposure.

At PND220, no significant increases in prostate inflammation were present (Table 4), however, the incidence, distribution, and severity of inflammation in the ventral prostate were decreased compared to that present at PND120. The lateral prostates were not different form control. There was no increase in incidence or severity of prostate inflammation over time following ATR exposure.

Myeloperoxidase Assay (MPO)-The MPO was assayed separately for PND120 and 220, and the values of the internal standards varied by less than 1% between runs. The positive and negative controls used in the assay resulted in the expected values. While total lateral prostate weights were increased at both PND120 and 220, MPO assay data showed that the inflammation in the left lateral prostates of offspring of treated dams was not different from controls at PND120 (Table 5). Even though, ATR-ATR males had 14% more MPO than control males, the difference lacked statistical significance. This increase was consistent with the observed increase in macroscopic alterations in that group of animals. A general increase in MPO concentration was noted in the lateral prostates within a treatment group between PND120 and 220. A significant decrease was seen between MPO concentrations in the ATR-ATR group and C-C group ($p<0.002$) at PND220. ATR-C animals had MPO concentrations similar to C-C while those in C-ATR groups were lower. This is inconsistent with the observations of visible abnormalities, but could possibly be explained by the use of the left lateral prostate and not the right in which the majority of visible abnormalities were observed at necropsy or because neutrophils were not always the predominant inflammatory cell present.

Serum Hormone Measurements- Serum hormone concentrations of total testosterone, androstenedione, estrone, and prolactin by RIA are presented in Table 6. There were no statistically significant differences in serum hormone concentration among the groups at PND120 for testosterone, androstenedione, estrone, and prolactin. However, there was a 35-40% increase in mean testosterone levels in C-ATR and ATR-ATR males when compared to control males, but these levels were within what is regarded to be a physiological range. The same males from these two groups also had slightly increased (7-10%) androstenedione

levels. These increases were not statistically different. All three ATR exposed groups had non-significant increases in estrone (10-20%) and prolactin (14-31%) levels.

By PND220 (Table 6), testosterone and estrone levels for all animals had decreased from PND120 levels, but ATR exposed animals' hormone levels still remained higher than controls. Testosterone levels in all ATR exposed groups were 20-60% higher than control levels. ATR-C and C-ATR males had slightly increased (26-29%) androstenedione levels vs. controls. Steroid hormone ratios, particularly those controlled by aromatase and 17-β hydroxysteroid dehydrogenase, were evaluated but no statistical differences were found at either time point. Prolactin levels in all ATR-exposed animals were significantly lower than those in the C-C group (33-55%). Prolactin levels in the ATR-ATR group were less than half of that measured in controls. These ATR-exposed animals also had enlarged pituitary glands at PND220 (Table 1).

Discussion

These studies demonstrated that a five-day exposure to 100 mg ATR/kg BW/d during late gestation resulted in effects present at seven months after the exposure period had ended in Long Evans rat offspring. The developing lateral prostate and anterior pituitary gland appeared to be sensitive to the effects of a brief prenatal or a residual lactational exposure to ATR. ATR from lactational exposure appeared to work in combination with *in utero* exposure to enhance delays in puberty, serum prolactin, pituitary weight, and lateral prostate weight compared to gestational exposure alone. Similar to what was observed for mammary gland (Rayner *et al*., 2004), continual exposure of the developing offspring to ATR is not required for altered gland development (prostate enlargement) or altered hormone status in later life. The males exposed to ATR only during gestation had enlarged prostates and anterior pituitary glands at seven months of age and decreased serum prolactin. Lactational exposure appeared to enhance the effects of gestational exposure resulting in prostate enlargement three months earlier. Lactational exposure only (C-ATR group) increased distribution of inflammation in the lateral prostate. The inflammation caused by ATR does not progress or become more severe over time. Therefore, it is not possible to predict from these data the full effects that perinatal ATR exposure may manifest in the adult, aged rat prostate.

Studies have shown that peripubertal ATR exposures delayed puberty (Stoker *et al*., 2000, Trentacoste *et al*., 2001) and lactational ATR exposures increased the incidence and severity of lateral prostate inflammation at PND120 (Stoker *et al*., 1999a). The present study also demonstrated a delay in puberty in males with combined gestational and lactational exposure. The female siblings of these males exposed gestationally and lactationally and

those exposed only through lactation also displayed delayed puberty. However, the females had increased weights at time of puberty (Chapter 4). It is possible that the lower body weight of the males contributed to the delayed puberty observed as suggested by Kennedy and Mitra (1967). Our data also agrees with previous reports (Laws *et al*., 2000; Stoker *et al.*, 2000) that the amount of atrazine needed to induce pubertal delays is much lower than the 100 mg/kg/d dose administered, because the 5 day dosing of the dams in our study ceased on GD19 and pups exposed to only the milk of those dams were affected (1 ½ day delay).

In our studies, we did observe lateral prostate inflammation in 120 day old control pups following suckling from dams with prenatal ATR exposure (potentially through lactational exposure). This effect was insignificant at the later time point suggesting the inflammation may be a transient effect, or that much longer exposures to increased circulating prolactin (and potentially steroid hormones) are needed to increase the incidence and severity of the effects on the prostate. Only males exposed through lactation had increased distribution of inflammation in the lateral prostate was consistent with Stoker *et al.,* (1999a). However, neither prenatal nor postnatal ATR exposure increased inflammation in the ventral prostate in our study and the combined exposure did not lead to increased inflammation of either prostatic lobe. The visual lateral prostate abnormalities noted at necropsy actually were increased by both pre-and postnatal ATR exposure (Table 2), but were located on the surface of the gland and may not have penetrated deep within the gland, as relatively small focal areas seem to be affected in the lateral prostate. The five slides per animal for pathological examination (step-sectioned through gland) covered a small portion of the entire gland and it is possible that we may have missed inflamed sites located deeper or more superficial in the gland. We also used a conservative approach in assessing

"inflammation" as 2 of the 5 slides had to reveal evidence of inflammation before we scored it as a positive. With that said, many reported prostate inflammation studies score a single slide for pathology. It is possible that not having both lateral prostate glands (one used for MPO and one for pathology) available for evaluation could have increased variability and caused the MPO concentrations and pathology to disagree. Judging by the visible lesions seen in our study, the lateral lobes of the prostate were not affected equally. Furthermore, the inconsistencies of a predominance of neutrophils in the affected prostate glands (at either timepoint) suggests that the inflammation may be more of a chronic state in some animals, and not an acute response as would be suggested by polymorphonuclear cells (neutrophils) at the inflammation site. This, too, would potentially cause a disagreement between the pathology results and MPO outcomes. Future studies should section and evaluate the entire prostate gland and not rely strictly on MPO if the response is not known to have an acute neutrophil response.

The present study on early life ATR exposure evaluated circulating hormone concentrations at about 4 and 7 months of life. We detected hormone levels that were both consistent and that differed from previous work. Stoker and colleagues (1999c) found no statistical differences in steroid hormones at PND120 when they examined male Wistar rats treated PND22-32 with pimozide, bisphenol A, or estradiol. They did find that prolactin levels in their treated animals were higher at PND29. By PND120, serum prolactin levels were not different between the groups. Males exposed to pimozide and bisphenol A also had increased lateral prostate weights at PND120. Perinatal methoxychlor exposure was shown to increase anterior pituitary prolactin levels (but not serum prolactin levels) and lateral prostate weights at PND90 (Stoker *et al.,* 1999b). In males exposed to ATR via

lactation, Stoker *et al*., (1999a) reported significantly lower concentrations of serum prolactin in PND120 males from dams exposed to 50 mg/kg ATR cotreated with ovine prolactin, but there were no lateral prostate differences among the treatment groups, suggesting that the addition of exogenous prolactin alleviated the adverse effects of ATR on pup prostate development. Stoker and colleagues (2000) found no differences in steroid or pituitary hormones at PND120 and no differences in lateral prostate weights at PND120 in male Wistar rats exposed to varying amounts of ATR during PND23-53. Our hormonal observations began at PND120, and no statistical differences were noted at that time, although trends for increase in testosterone and prolactin were noted. However by PND220, there was a decrease in serum prolactin and coincidently an increase in anterior pituitary gland weight in males born to ATR treated dams. While ATR has not been classified as an estrogen mimic, it is considered to be an endocrine disruptor and has been shown to suppress prolactin and luteinizing hormone (Cooper *et al.,* 2000). ATR may disrupt placental and early postnatal hormones leading to permanent changes in early prostate development resulting in growth with age, as observed by the lateral prostate weight increases in the present study.

The results of the present study suggest that the developing lateral prostate glands in Long Evans rata are sensitive to gestational, and lactational ATR exposure. A combination of pre- and early postnatal ATR exposures delayed puberty, enhanced lateral prostate gland effects, and decreased serum prolactin. The exposure parameters responsible for the pubertal outcomes are consistent with what we previously reported for the female siblings (Chapter 4). However, the males may have been more sensitive to ATR as they also exhibited slight decrements in body weight at the time of puberty that was not observed in the female pups.

Table 1. Effect of 100 mg/kg ATR* on reproductive organ weights at PND120 and PND220

PND120	C-C[1]	ATR-C	C- ATR	ATR-ATR
Body Wt(g)	661.6 ± 17.3	623.4 ± 18.5	605.7 ± 13.9[a]	616.0 ± 18.3
Pituitary(mg)	10.08 ± 0.66	9.65 ± 0.71	9.64 ± 0.49	10.34 ± 0.34
Left Testis(g)	1.99 ± 0.04	1.98 ± 0.03	1.87 ± 0.08	1.88 ± 0.07
Right Testis(g)	1.92 ± 0.04	1.92 ± 0.03	1.87 ± 0.04	1.85 ± 0.07
Seminal Vesicle Wet(g)	1.64 ± 0.25	1.45 ± 0.10	1.43 ± 0.08	1.58 ± 0.07
Seminal Vesicle Dry(g)	0.728 ± 0.04	0.698 ± 0.04	0.700 ± 0.02	0.774 ± 0.03
Ventral Prostate(mg)	524.0 ± 42.4	469.9 ± 33.6	451.1 ± 28.0	532.3 ± 32.9
PND220				
Body Wt(g)	777.3 ± 26.6	703.5 ± 23.5[ab]	804.1 ± 31.5	739.3 ± 10.8
Pituitary(mg)	8.41 ± 0.98	10.38 ± 1.05[c]	10.52 ± 0.81	12.9 ± 0.72[de]
Left Testis(g)	1.97 ± 0.06	1.89 ± 0.07	2.01 ± 0.06	2.10 ± 0.07
Right Testis(g)	1.92 ± 0.06	1.85 ± 0.07	1.99 ± 0.05	2.03 ± 0.07
Seminal Vesicle Wet(g)	1.67 ± 0.12	1.74 ± 0.09	1.92 ± 0.15	1.86 ± 0.10
Seminal Vesicle Dry(g)	0.775 ± 0.04	0.851 ± 0.03	0.944 ± 0.05	0.819 ± 0.05
Ventral Prostate(mg)	545.5 ± 26.9	576.5 ± 23.8	579.6 ± 44.1	525.3 ± 56.3

ATR= atrazine, PND= post natal day. Data presented as dam mean ± SE. [1]Dam-Milk source.
PND120- Dam N=5-6, with n= 10 offspring per exposure group.
PND220- Dam N=4-5, with n = 8-10 offspring per exposure group.
[a] Significant treatment effect by ANOVA; p<0.05 vs. C-C.
[b] Significant treatment effect by ANOVA; p<0.01 vs. C-ATR.
Analysis indicated is shown for BW as covariate.
[c] Significant treatment effect by ANOVA; p<0.03 vs. C-C.
[d] Significant treatment effect by ANOVA; p<0.01 vs. C-C.
[e] Significant treatment effect by ANOVA; p<0.03 vs. C-ATR

Table 2. Visual evidence of prostate abnormalities at necropsy

Dam-Milk Source	PND 120	PND220
C-C	0/10	0/8
ATR-C	0/10	2/9
C-ATR	4/10	4/10
ATR-ATR	7/10[a]	5/9[b]

Data presented as # of animals exhibiting prostate abnormalities/ total # of animals per group.
PND120- Dam N=5-6, with n= 10 offspring per exposure group.
PND220- Dam N=4-5, with n = 8-10 offspring per exposure group.
Analysis of abnormalities- 2 sided p- values
[a] p=0.003 vs. C-C &C-ATR.
[b] p =0.03 vs. C-C.

Table 3. Prostate pathology on PND120

Lateral prostate inflammation:

Group*	Incidence	Distribution (total)	Distribution (affected)	Severity (total)	Severity (affected)	Neutrophils	Total Incidence Inflammation
C-C	0/10	0	0	0	0	0	8/10
ATR-C	0/10	0	0	0	0	0	8/10
C-ATR	3/10	0.5 ± 1.0[a]	1.7 ± 1.2	1.1 ± 2.1	3.7 ± 2.3	2/3	7/10
ATR-ATR	1/10	0.1 ± 0.3	1.0	0.5 ± 1.5	5.0	1/1	6/10

Ventral prostate inflammation:

	Incidence	Distribution (total)	Distribution (affected)	Severity (total)	Severity (affected)	Neutrophils
C-C	8/10	1.1 ± 0.07	1.4 ± 0.5	1.2 ± 0.9	1.5 ± 0.7	3/8
ATR-C	8/10	1.7 ± 1.3	2.1 ± 1.0	1.1 ± 1.1	2.0 ± 0.8	4/8
C-ATR	7/10	1.4 ± 1.2	2.0 ± 0.8	1.3 ± 0.9	1.9 ± 0.4	2/7
ATR-ATR	6/10	1.1 ± 1.1	1.8 ± 0.8	1.0 ± 1.1	1.7 ± 0.8	1/6

*** Group= Dam-Milk Source Data presented as dam mean ± SE. PND120- Dam N=5-6, with n= 10 offspring per exposure group.**
All significant effects vs. Control. Total Incidence=lateral and ventral lobes. [a] Significant treatment effect by t-test; $p<0.05$.

Table 4. Prostate pathology on PND220

Lateral prostate inflammation: Group*	Incidence	Distribution (total)	Distribution (affected)	Severity (total)	Severity (affected)	Neutrophils	Total Incidence Inflammation
C-C	1/8	0.1 ± 0.4	1.0	0.5 ± 1.4	4.0	1/1	2/8
ATR-C	2/9	0.3 ± 0.7	1.5 ± 0.7	0.9 ± 1.8	4.0 ± 1.4	2/2	4/9
C-ATR	3/10	0.2 ± 0.6	2.0	0.5 ± 1.6	5.0	1/3	2/10
ATR-ATR	2/9	0.2 ± 0.4	1.0	0.8 ± 1.8	4.0	2/2	4/9
Ventral prostate inflammation:							
	Incidence	Distribution (total)	Distribution (affected)	Severity (total)	Severity (affected)	Neutrophils	
C-C	1/8	0.1 ± 0.4	1.0	0.1 ± 0.4	1	1/1	
ATR-C	2/9	0.2 ± 0.4	1.0	0.4 ± 1.0	2.0 ± 1.4	0/2	
C-ATR	1/10	0.1 ± 0.3	1.0	0.3 ± 0.9	3	1/1	
ATR-ATR	3/9	0.4 ± 0.7	1.3 ± 0.6	0.9 ± 1.4	2.6 ± 0.6	1/3	

*** Group= Dam-Milk Source Data presented as dam mean ± SE. PND220- Dam N=4-5, with n = 8-10 offspring per exposure group. Total Incidence=lateral and ventral lobes.**

Table 5. Concentration (ng/ml) of myeloperoxidase in left prostate at PNDs 120 and 220

	C-C[1]	ATR-C	C- ATR	ATR-ATR
PND120	44.5 ± 2.75	41.3 ± 1.83	45.3 ± 2.50	51.9 ± 3.61
PND220	224.1 ± 29.1	228.7 ± 35.6	155.3 ± 22.8	84.7 ± 16.2[a]

Data presented as dam mean ± SE. [1]Dam-Milk source. PND120- Dam N=5-6, with n= 10 offspring per exposure group. PND220- Dam N=4-5, with n = 8-10 offspring per exposure group. [a] Significant treatment effect by ANOVA; p<0.01 vs. C-C.

Table 6. Serum hormone concentration at PND120 and PND220

PND120	C-C[1]	ATR-C	C- ATR	ATR-ATR
Testosterone(ng/ml)	1.00 ± 0.17	1.04 ± 0.24	1.41 ± 0.29	1.35 ± 0.31
Androstenedione(ng/ml)	0.360 ± 0.04	0.353 ± 0.04	0.395 ± 0.05	0.386 ± 0.04
Estrone(pg/ml)	43.5 ± 3.01	52.1 ± 4.82	50.4 ± 4.30	47.9 ± 3.80
Prolactin(ng/ml)	2.97 ± 0.39	4.10 ± 0.71	3.31± 0.34	3.38 ± 0.39
PND220				
Testosterone(ng/ml)	0.613 ± 0.18	0.982 ± 0.22	0.737 ± 0.14	0.819 ± 0.15
Androstenedione(ng/ml)	0.318 ± 0.05	0.410 ± 0.03	0.400 ± 0.04	0.315 ± 0.05
Estrone(pg/ml)	16.9 ± 2.21	20.7 ± 2.79	21.4 ± 2.96	25.1 ± 2.91
Prolactin(ng/ml)	5.72 ± 0.86	3.81 ± 0.38[a]	3.86 ± 0.55[a]	2.55 ± 0.19[b]

Data presented as dam mean ± SE. [1]Dam-Milk source. PND120- Dam N=5-6, with n= 10 offspring per exposure group. PND220- Dam N=4-5, with n = 8-10 offspring per exposure group. [a] Significant treatment effect by ANOVA; p<0.02 vs. C-C. [b] Significant treatment effect by ANOVA; p<0.01 vs. C-C.

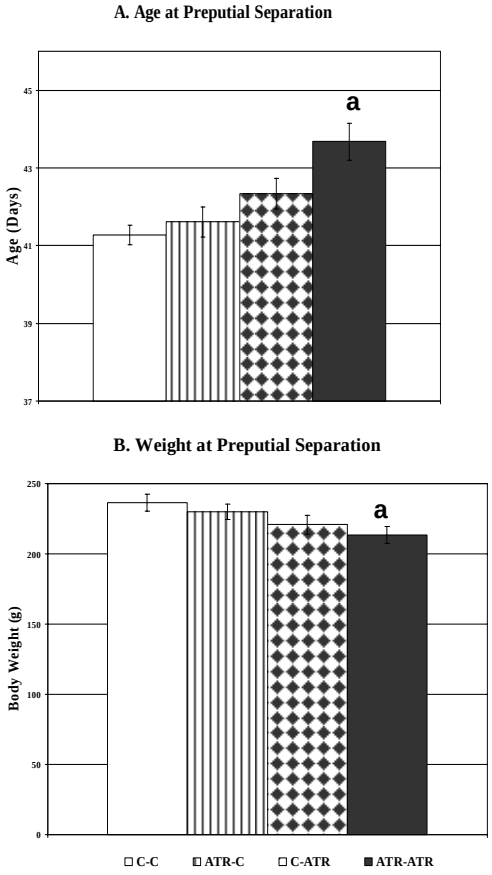
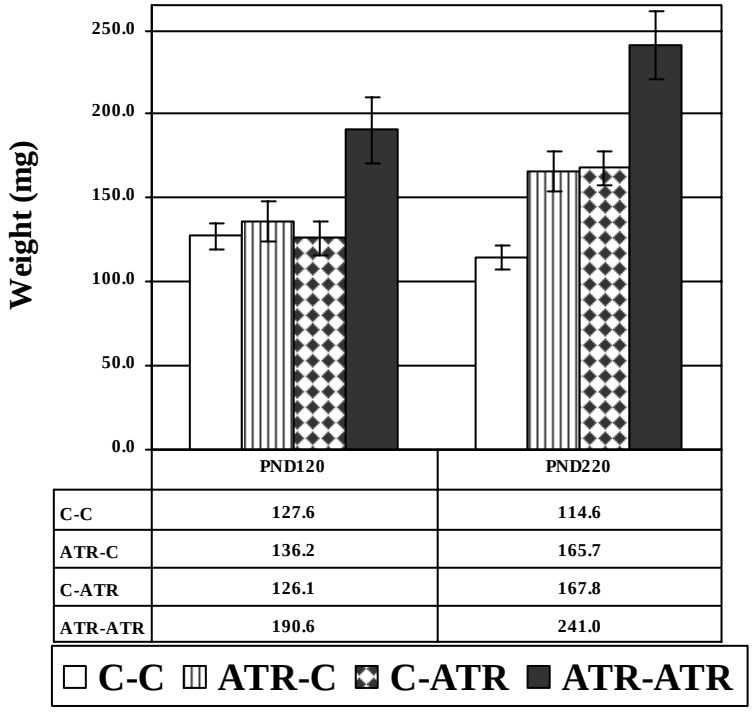


Figure 1. Effect of gestational exposure to 100 mg/kg BW/day on age and weight at time of preputial separation (PPS). (A) Age (days) at the time of PPS. Data presented as dam mean ± SE. Groups listed as dam-milk source. Significant treatment effect by ANOVA (LSM). [a]Different from C-C, $p<0.0001$. (B) Dam mean ± SE of body weight (g) at the time of PPS. [a]Different from C-C, $p<0.01$.

Lateral Prostate Weight (PND120 & PND220)



	PND120	PND220
C-C	127.6	114.6
ATR-C	136.2	165.7
C-ATR	126.1	167.8
ATR-ATR	190.6	241.0

Figure 2. Total lateral prostate weight at PNDs 120 & 220. Data presented as dam mean $\pm$ SE (mg). Groups listed as dam-milk source. Significant treatment effect by ANOVA (LSM). [a]Different from C-C, ATR-C, and C-ATR, $p<0.001$. [b] Different from C-C $p<0.03$. [c]Different from C-C, ATR-C, and C-ATR, $p<0.03$.

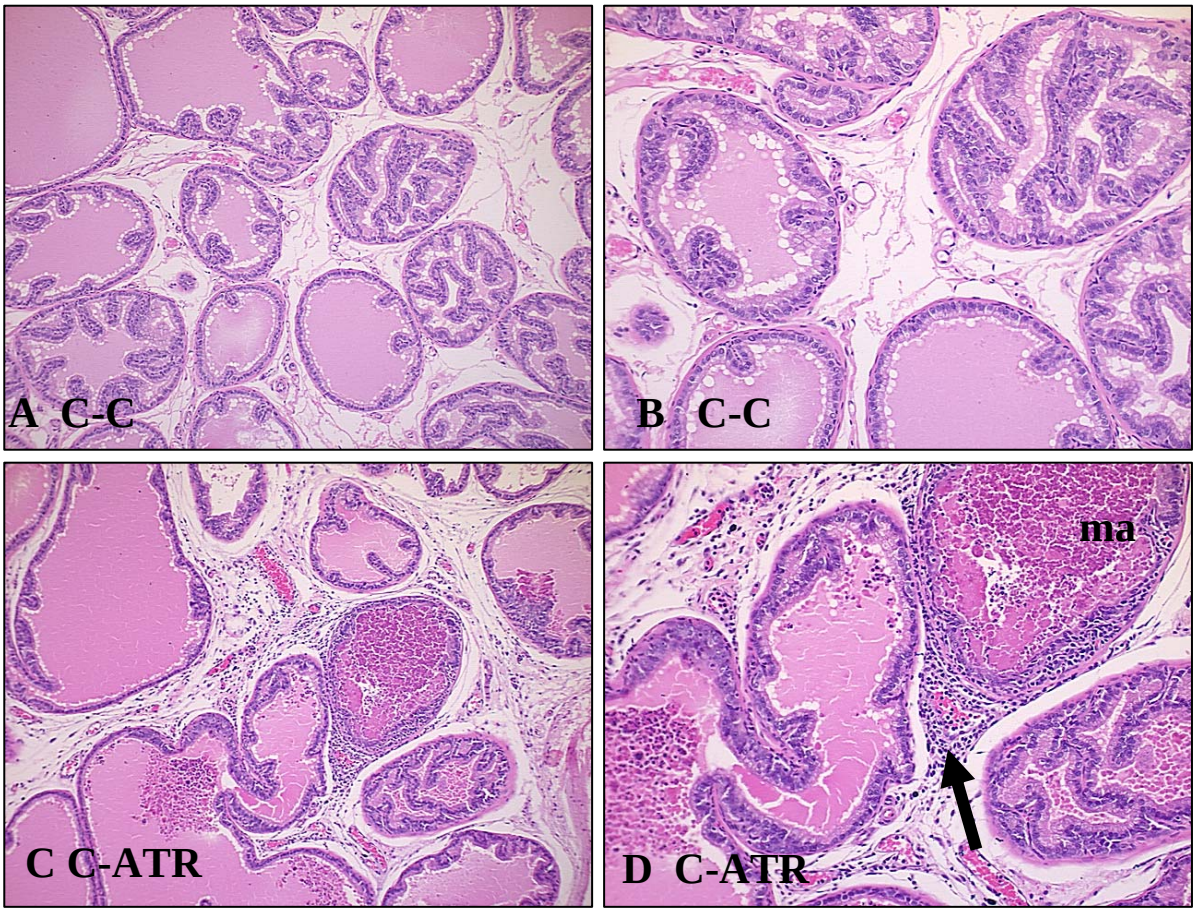


Figure 3. Histological sections of lateral prostates at PND120. Groups listed as dam-milk source. (A, B) C-C offspring displaying few inflammatory cells. (C, D) C-ATR offspring found to have statistically significant distribution and severity of inflammation. Note the infiltration (arrow) of inflammatory cells and micro-abscess (ma) formation. A, C at 100X; B, D at 200X magnification.

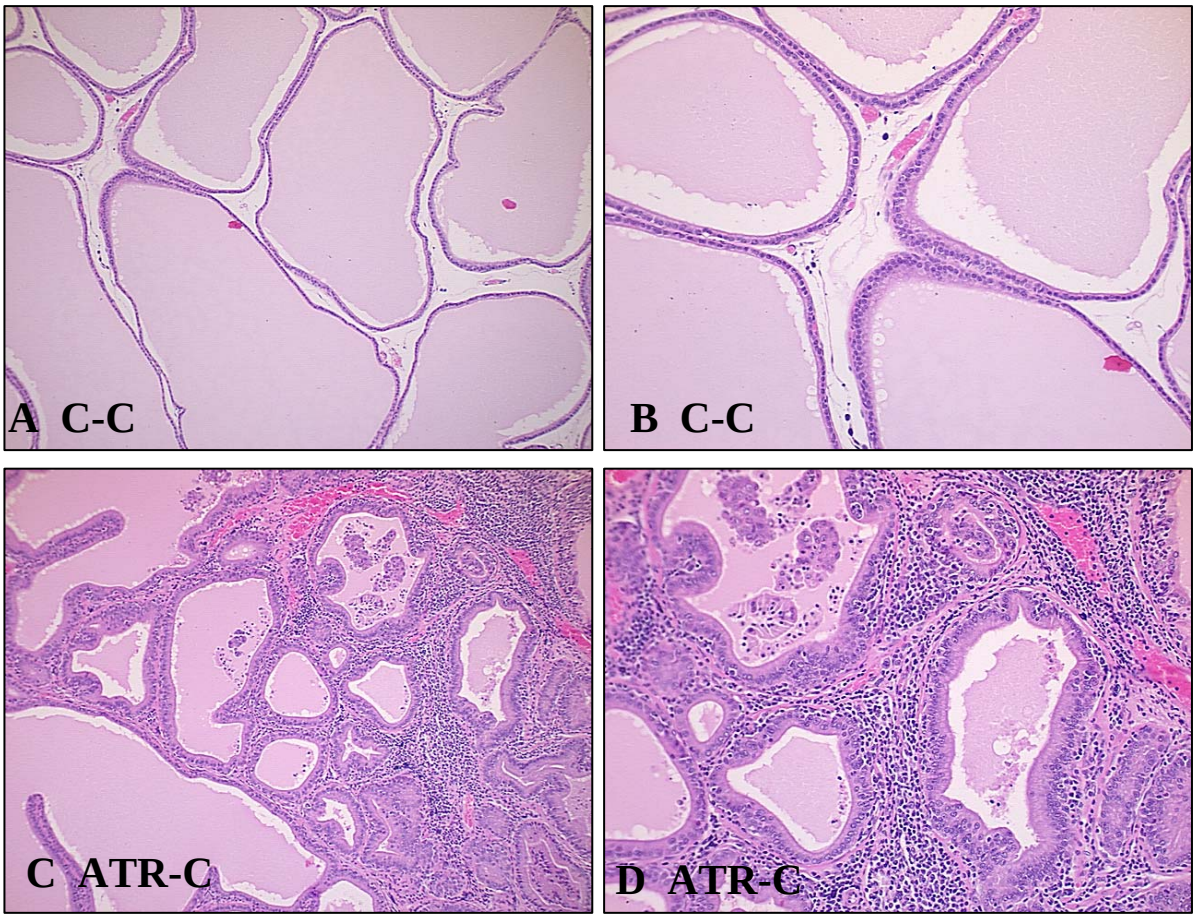


Figure 4. Histological sections of ventral prostates at PND120. (A, B) C-C offspring. (C, D) ATR-C offspring. The ATR-C prostate shown represents severe inflammation given a grade of 3 in this prostatic lobe. A, C at 100X; B, D at 200X magnification.

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