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Adverse Effects of Prenatal Exposure to Atrazine During a Critical Period of Mammary Gland Growth

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Prenatal exposure to 100 mg/kg atrazine (ATR) delays mammary gland (MG) development in resulting female offspring of Long-Evans rats. To determine if the fetal MG was sensitive to ATR effects during specific periods of development, timed-pregnant dams (n = 8/group/block) were dosed for 3- or 7-gestation day (GD) intervals (GD 13–15, 15–17, 17–19, or 13–19) with 100 mg ATR/kg/day or vehicle (C), and their offspring were evaluated for changes. Mammary glands taken from pups prenatally exposed to ATR displayed significant delays in epithelial development as early as postnatal day (PND) 4 compared to C, with continued developmental delays at later time points that varied by time of exposure. However, the most persistent and severe delays were seen in the GD 17–19 and GD 13–19 ATR exposure groups, demonstrating statistically similar growth retardation. Because MG developmental deficits persisted into adulthood, we hypothesized that ATR-exposed animals may have had difficulties nursing their young. Females exposed prenatally to either ATR (as defined) or C (n = 4 litters/group) were bred, and the resulting F_2 offspring from GD 17–19 and GD 13–19 exposure groups were significantly smaller in body weight (BW) than C. In a separate study, it was determined that ATR (25–100 mg/kg), delivered from GD 15–19, did not decrease fetal body weights on GD 20, even though the higher doses significantly decreased weight gain of the dosed dams. These data suggest that the consequences of brief ATR exposure during a critical period of fetal MG development (GD 17–19), are both delayed MG development of the offspring and inadequate nutritional support of F_2 offspring, resulting in adverse effects on pup weight gain.

Key Words: **atrazine; mammary gland; critical period; lactation.**

INTRODUCTION

Atrazine, (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, 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, sorghum, and fruit crops (Gianessi, 1998).

Our previous work (Rayner *et al.*, 2004) demonstrated that a 5-day exposure to 100 mg/kg ATR during gestation days (GD) 15–19 in Long-Evans hooded rats (LE) delayed vaginal opening (VO), a sign of puberty, but did not affect estrous cyclicity or serum hormone concentrations in female offspring. This 5-day gestational exposure also delayed mammary gland (MG) growth and development in female offspring, whether or not the animals were exposed to ATR prenatally only or continued to nurse from ATR-exposed dams. However, the delayed growth and development of the MG was seen in pups as early as postnatal day (PND) 4, prior to any potential confounding hormonal effects of puberty, and were independent of pup BW (maximum decrease of 5.7% seen only in pups exposed to ATR both prenatally and potentially via nursing). The delays in development were persistent into adulthood, and were most severe in the animals that were exposed to ATR prenatally and that continued to nurse from an ATR-exposed dam.

Along with other reproductive tissues, MG development begins *in utero*. The mammary bud appears in mice around GD 10 or 11 (Imagawa *et al.*, 1990), and slightly later in rats. Between GD 12 (mice) and GD 16 (rats), the bud increases in size, and the mesenchyme around the bud begins to differentiate (Hovey *et al.*, 2002; Knight and Sorensen, 2001). From GD 16 to birth (mice and rats), the epithelial cells proliferate rapidly to form the beginning of the ductal tree. Also rapidly proliferating at this time is the mammary fat pad, which forms the support structure for the branching ducts (Hovey *et al.*, 2002; Imagawa *et al.*, 1990). The growth of the gland is typically isometric, growing at the same rate as the body (Borellini and Oka, 1998), but shortly before puberty the gland undergoes allometric growth, growing at a rate two to three times faster than body growth rate (Borellini and Oka, 1998; Hovey *et al.*, 2002), until terminal ducts (end structures that are a single cell layer thick and static) and lobules have formed

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(Daniel and Silberstein, 1987). The functional differentiation of the gland takes place during pregnancy.

Several studies have shown that developing MG are sensitive to toxicant exposure. Fenton *et al.* (2002) showed that the MG of female offspring of Long-Evans dams exposed *in utero* to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) on GD 15 displayed persistently delayed development. The glands of female offspring at several developmental time points had not migrated through the fat pad, lateral branching was reduced, and terminal end-buds (rapidly dividing cells in tear-drop–shaped structures) were present for extended lengths of time. Foster *et al.* (2004) treated Sprague-Dawley dams with a mixture consisting of organochlorines, chlorinated benzenes, and metals at the acceptable daily intake level on GD 9–16, with or without postnatal genistein. Female offspring receiving both the mixture and genistein had increased female MG morphological alterations, including calcifications, epithelial hyperplasia, and cystic dilation, compared to the control and mixture groups. Bisphenol A (BPA) was shown to alter the development of CD-1 mouse MG when exposed beginning on GD 9 and continuing throughout the pregnancy (Markey *et al.*, 2001). Glands removed 1 month after birth from offspring of dams treated with 25 μg/kg BPA showed increased ductal elongation, whereas glands from offspring of dams treated with 250 μg/kg BPA demonstrated decreased elongation as compared to control offspring glands (possibly because of different signaling components in the low-dose effect). By 6 months of age, both groups had significantly larger ductal and alveolar structures than controls. These studies, taken together, demonstrate that MG of rats and mice can be sensitive to the effects of environmental agents during the latter half of prenatal development.

A 5-day exposure to 100 mg/kg/day ATR during GD 15–19 in Long-Evans rats delayed MG growth and development in female offspring (Rayner *et al.*, 2004). To determine if there is a 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. 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. These persistent effects of prenatal atrazine exposure result in decreased weight gain in litters born to atrazine-exposed females.

METHODS

Animals. Timed-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. The work reported here was conducted in two separate studies, with dosing solutions for each study described below.

- *Critical period study.* 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 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/day) was chosen because of the consistent reproductive endpoint effects observed in previous studies (Laws *et al.*, 2000; Rayner *et al.*, 2004; Stoker *et al.*, 1999).

Fetal weight study. Atrazine was prepared as a suspension in 1.0% methyl cellulose in distilled water. Timed-pregnant rats were treated with 0 (vehicle), 25, 50, or 100 mg ATR/kg BW by oral gavage in a 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/day). Control dams were dosed on gestational days (GD) 13–19, and dams receiving ATR were dosed either on GD 13–15, GD 15–17, GD 17–19, or GD 13–19. Litters were weighed and randomly equalized to 10 pups (6 females, 4 males) on PND 4. At weaning, PND 22 or PND 25, animals were weighed and separated into unisex sibling groups, 2 female rats per cage. Male pups were used in a separate study unrelated to this one. Females, at least 2/dam and 4 dams/group/block were sacrificed on PND 4 (from remaining pups after equalization of litters), PND 22, PND 25, PND 33, PND 46, and PND 67. The second block was extended to PND 67 because of persistent effects seen in Block 1 mammary glands (ended on PND 46). Beginning on PND 68, sibling females ($n = 4$/group) of those sacrificed on PND 67 and exhibiting normal estrous cycles were bred to control males. Second generation litters were weighed and randomly equalized to 10 pups on PND 4. On PND 11 of the 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.* Thirty-two pregnant LE dams were treated with vehicle (control), 25, 50, or 100 mg/kg ATR daily ($n = 8$ dams/treatment) on GD 15–19 as previously described (Rayner *et al.*, 2004). On GD 20, 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 PND 29, 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 PND 37 and continued until PND 67 to determine the effect of prenatal ATR exposure on early cyclicity patterns following VO. Vaginal smears were read wet on an American Optical low-power light microscope (100×) for the presence of leukocytes (metestrus/diestrus), nucleated epithelium (proestrus), or cornified epithelial cells (estrus) to determine cyclicity patterns, 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. Animals demonstrating irregular cyclicity were defined as those not presenting at least two consecutive normal cycles between PND 37 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 PND 33, 46, or 67, ≥2 females/dam were weighed and decapitated. On PND 33 ($n = 6$ dams/treatment group), MG were removed for whole mounts from female offspring that had undergone VO in all dose groups, so that differences in MG development could be evaluated separately from other facets of puberty. Trunk blood was collected and centrifuged for 30 min at 3000 rpm (4°C) for serum. At PND 46 ($n > 7$ dams/group) mammary glands were removed for whole mounts. On PND 67 ($n > 4$ dams/group), trunk blood was collected and centrifuged for 30 min at 3000 rpm (4°C) for serum from animals killed between 0800–1300 h. The pituitary gland, ovaries, and uterus (wet) were removed and weighed. Mammary glands were removed for whole mounts. Normal cycling female siblings remaining after PND 67 were retained for breeding experiments.
- *Fetal weight study.* On GD 20, dams ($n > 7$/group) were decapitated, and trunk blood was collected and centrifuged as above for serum. The 4th and 5th 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. 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 PND 4, 22, 25, 33, 46, and 67 (2–4 pups/dam). Mammary glands from second generation pups were removed and stained on PND 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 PND 46 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; procedure available upon request) within an age group, by two individual scorers without knowledge of treatment. 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 PND 33 and PND 67, and from dams at PND 11 (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 was measured 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 PND 10. On PND 11, dams were removed, weighed, and placed in clean individual cages with food and water *ad libitum.* Entire litters were weighed, and pups allowed to remain in their own nests. 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 min, 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 MG were removed from female pups for whole mount analysis. The uterus of dams was removed and implantation sites were counted. Male pups were euthanized.

Statistical analysis. Dam means (pups/dam; litter as unit) were calculated for body and tissue weights, MG scores, VO day, and serum hormones. Means and adjusted means relative to body weight were calculated for organ weights. Body weights, MG 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 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 MG 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 5-day ATR exposure (100 mg/kg BW/day) during late gestation could delay MG development, as well as puberty.

Dam weight was recorded throughout the dosing periods, and weight gain was compared among groups (Table 1). During the GD 13–15 exposure period, control dams (vehicle) gained an average of 20.4 ± 3.4 g. Dams exposed to 100 mg/kg BW/day ATR during GD 13–15 and the first 3 days of GD 13–19 gained no weight, significantly less than control dams, $p < 0.0001$. Dams dosed with ATR GD 15–17 gained significantly less weight than control dams, with weight gain reduced 82%, ($p < 0.0001$), and ATR GD 13–19 dams had a 35% reduced weight gain, ($p < 0.0007$) on the middle 3 days of that exposure. In the final dosing period, dams in the ATR GD 17–19 group had a 90% reduction in weight gain ($p < 0.0001$) compared to control dams. However, those exposed to ATR GD 13–19 exhibited only a 26% reduction in weight gain during the GD 17–19 period, ($p < 0.0403$). During the entire dosing period GD 13–19, control dams gained a total of 78.2 ± 6.1 g and ATR-exposed dams less than half of that amount ($p < 0.0001$), with most of that

TABLE 1
Critical Period Study: Effect of 100 mg/kg Atrazine (ATR) on Maternal Weight Gain (g)

		ATR exposure period			
Group	*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 ± 1.8[a]			
ATR GD15–17	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[b]	22.7 ± 2.0[c]	40.1 ± 3.4[a]

Note: n = number of dams on gestation days (GD) shown.
All significant effects versus Control. Data presented as dam mean ± SE.
[a]Significant treatment effect by ANOVA (LSM); $p < 0.0001$.
[b]Significant treatment effect by ANOVA (LSM); $p < 0.007$.
[c]Significant treatment effect by ANOVA (LSM); $p < 0.0403$.

TABLE 2
Treatment Effects on Dam Weight Gain and Fetus Outcome on GD 20[a]

Group	*n*	Maternal weight gain (g)	Litter means	Resorbed fetuses	Dead fetuses[b]
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 ± 0.16	0.12 ± 0.12
50 mg/kg ATR	8	29.7 ± 6.7[c]	13.9 ± 0.6	0.43 ± 0.20	0 ± 0.0
100 mg/kg ATR	7	19.0 ± 3.2[d]	11.1 ± 1.5	1.62 ± 1.03[e,f]	0.25 ± 0.16

Note: ATR = atrazine; n = number of dams.
[a]Weight gain during GD 15–19 dosing period.
[b]Dead but not resorbed fetuses.
All significant effects versus. Control except where noted. Data presented as litter mean ± SE.
[c]Significant treatment effect by ANOVA (LSM); $p < 0.0024$.
[d]Significant treatment effect by ANOVA (LSM); $p < 0.001$.
[e]Significant treatment effect by Fisher's exact test; two-sided $p < 0.0009$.
[f]Significant treatment effect by Fisher's exact test; two-sided $p < 0.0082$ vs 25 mg/kg and two sided $p < 0.0251$ versus 50 mg/kg.

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 during all periods tested.

The combined mortality rates from PND 4 (after equalizing litters) to weaning for both blocks were 24.6% for control, 33.2% for GD 13–15, 40.5% for GD 15–17, 30.0% for GD 17–19, and 26.3% for GD 13–19. 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. Furthermore, 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 GD 15–19 to 100 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. Atrazine-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 fetal body weight.

Dam weight was recorded daily throughout the dosing periods, and weight gain was compared among groups. During the exposure period GD 15–19, control dams gained an average of 51.2 ± 3.0 g (Table 2). Dams in the 25, 50, 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 groups 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 GD 20 (24 h 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 ± 2.22 C; 6.40 ± 0.78 ATR 25 mg/kg; 8.44 ± 1.84 ATR 50 mg/kg; and 4.78 ± 0.48 ATR 100 mg/kg), although the highest ATR dose reduced serum PRL to half that in controls.

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 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, $p < 0.0009$ (Table 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 100 mg/kg group were also significantly greater than the means for the 25 mg/kg group (2 sites total) and the 50 mg/kg (3 sites total) group, $p < 0.0082$ and $p < 0.0251$. 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 (Fig. 1) and no differences were found at GD 20. 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

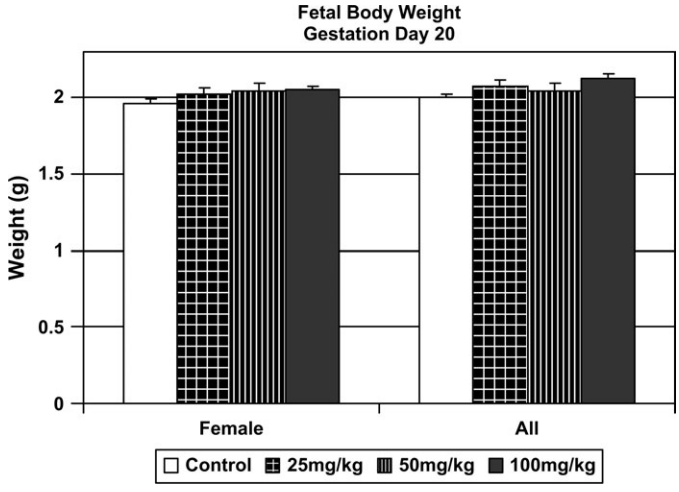

FIG. 1. Litter mean ($n > 7$/group) body weights (g) of GD 20 pups exposed to increasing concentrations of atrazine during GD 15–19 (25, 50, and 100 mg/kg). Data are presented as litter mean ± SE. Weights at this time point were not reduced compared to control in any dose group.

points. At PND 4, there were no differences in the body weights of female offspring (10.2 ± 0.2 g C, 10.0 ± 0.2 g GD 13–15, 10.2 ± 0.2 g GD 15–17, 9.7 ± 0.2 g GD 17–19, and 10.4 ± 0.2 g GD 13–19). There were also no differences in body weight at PND 22, 25, 33, or 46. In fact, offspring from GD 13–19–exposed dams weighed about the same as control offspring at PND 4 (1.1% change vs control), PND 22 (1.7%), PND 25 (1.3%), PND 33 (7.0%), and PND 46 (3.5%). However, at PND 67, both GD 15–17 and GD 13–19 animals weighed significantly less than control, $p < 0.01$.

Vaginal opening and estrous cyclicity were evaluated as physical signs of female reproductive development (Fig. 2). Body weight at time of VO was not different among the treatment groups (panel A). Vaginal opening occurred in control animals at PND 32.6 ± 0.33 (panel B). The offspring exposed to ATR on GD 13–19 displayed a significant delay in VO (34.5 ± 0.36, $p < 0.0004$), similar to that seen previously (Rayner *et al.*, 2004). Vaginal opening was not significantly delayed in any other dose group (33.5 ± 0.41 GD 13–15; 32.9 ± 0.31 GD 15–17; and 33.2 ± 0.36 GD 17–19).

Estrous cyclicity patterns of the female offspring were observed from PND 37 to PND 67. 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 fewer 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 PND 4, 22, 25, 33, 46, and 67 and examined to determine if epithelial development of the MG was affected by gestational ATR exposure.

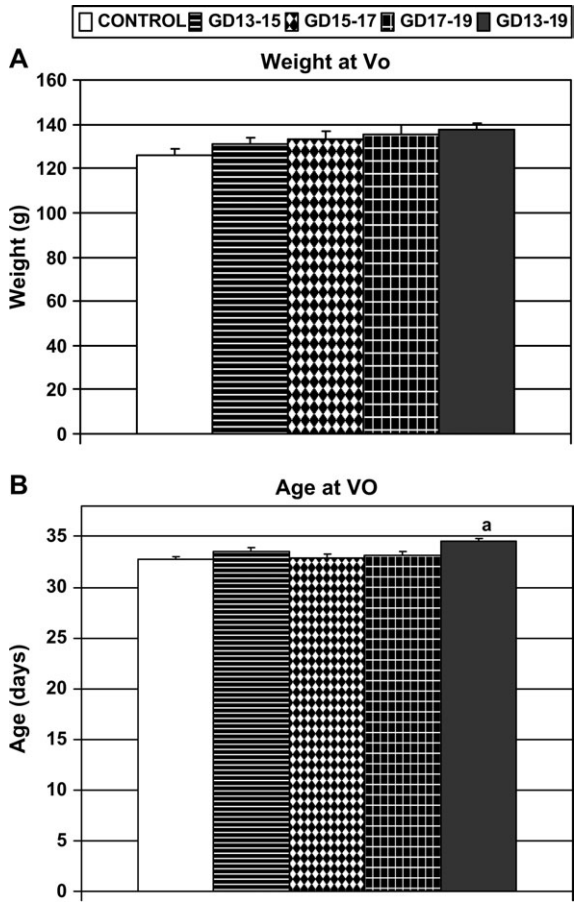

FIG. 2. Effect of gestational exposure to 100 mg atrazine/kg BW/day on female pubertal body weights and age at time of vaginal opening (VO). (A) Litter mean ($n > 4$ litters) body weight (g) at the time of vaginal opening. (B) Litter mean age (days) at the time of vaginal opening. Data are presented as mean ± SE. [a]Significant treatment effect by ANOVA (LSM) and significantly different from control ($p < 0.0004$).

Stained epithelia were measured (area [mm^2] and length [mm]) to observe differences in outgrowth into the fat pad (Table 3). Mammary gland development in female offspring was scored through whole mount analysis (Table 4, Fig. 3A).

The area (mm^2) of the mammary gland on PND 4 in all 3-day ATR-exposed groups was significantly smaller, $p < 0.0008$, compared to control offspring (22.5 ± 2.7 mm^2), and glands from the GD 13–19 exposure group measured 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 GD 15–19 (GD 15–17, 17–19) and GD 13–19 were smaller and developmentally delayed in branching and ductal structures compared to glands taken from control offspring (Fig. 3, Table 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) caused by treatment.

Weaning occurred at either PND 22 (Block 2) or PND 25 (Block 1), and MG were taken from exposed offspring of

TABLE 3
Effect of 100 mg/kg ATR on 4th Mammary Gland Areas (mm^2) at PND 4–33 and Epithelial Length (mm) at PND 46

Day	Control	GD 13–15	GD 15–17	GD 17–19	GD 13–19
PND 4	22.5 ± 2.70	14.3 ± 1.20[a]	14.0 ± 1.34[b]	12.7 ± 0.843	10.9 ± 1.13[c]
PND 22	77.4 ± 9.01	69.2 ± 13.0	43.3 ± 2.89[d]	48.5 ± 10.4[e]	30.6 ± 6.82[f]
PND 25	126.3 ± 12.3	85.4 ± 5.20[g]	75.5 ± 8.26[h]	67.6 ± 6.19[c]	57.7 ± 10.9[c]
PND 33	192.9 ± 11.0	175.8 ± 9.76	195.6 ± 11.5	175.2 ± 9.14	152.2 ± 8.74
PND 46	52.2 ± 0.620	49.2 ± 1.17	50.0 ± 0.798	52.4 ±1.80	48.8 ± 1.28

Note: ATR = atrazine. PND = postnatal day. Dam $n > 8$,with ≥ 5 offspring per exposure group at each time.
All significant effects versus Control. Data presented as dam mean ± SE.
[a]Significant treatment effect by ANOVA (LSM); $p < 0.0008$.
[b]Significant treatment effect by ANOVA (LSM); $p < 0.0005$.
[c]Significant treatment effect by ANOVA (LSM); $p < 0.0001$.
[d]Significant treatment effect by ANOVA (LSM); $p < 0.0140$.
[e]Significant treatment effect by ANOVA (LSM); $p < 0.0335$.
[f]Significant treatment effect by ANOVA (LSM); $p < 0.0012$.
[g]Significant treatment effect by ANOVA (LSM); $p < 0.0025$.
[h]Significant treatment effect by ANOVA (LSM); $p < 0.0002$.

equivalent BW. The area of the fourth gland remained significantly smaller in exposed offspring at PND 22, $p < 0.03$ (Table 3), with the exception of GD 13–15 offspring, but the distances between the fourth and fifth glands were not different from control. The mammary glands of GD 17–19 and GD 13–19 offspring displayed fewer terminal end buds and lateral branches, and they had not migrated as far through the fat pad as controls (Table 4, Fig. 3B). The area of the fourth gland remained significantly smaller in ATR-exposed offspring at PND 25 (Table 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 mammary branching was sparse in appearance with fewer lateral branches than control (Table 4, Fig. 3A).

TABLE 4
Effect of 100 mg/kg ATR Evaluated by Subjective Scoring of Mammary Gland Development

Day	Control	GD 13–15	GD 15–17	GD 17–19	GD 13–19
PND 4	3.2 ± 0.25	2.8 ± 0.18	2.3 ± 0.20[a]	1.9 ± 0.20[b]	2.1 ± 0.10[b]
PND 22	3.2 ± 0.32	2.7 ± 0.39	2.4 ± 0.19	2.0 ± 0.08[c]	1.6 ± 0.17[a]
PND 25	3.3 ± 0.18	2.7 ± 0.15[d]	2.4 ± 0.17[e]	2.2 ± 0.17[f]	2.2 ± 0.24[g]
PND 33	3.5 ± 0.11	3.0 ± 0.11[g]	2.7 ± 0.07[b]	2.7 ± 0.10[b]	2.6 ± 0.05
PND 46	3.7 ± 0.14	3.0 ± 0.11[b]	3.0 ± 0.09[f]	2.9 ± 0.07[b]	2.7 ± 0.10[b]
PND 67	3.4 ± 0.06	2.8 ± 0.21[h]	2.3 ± 0.24[i]	2.6 ± 0.22[j]	2.4 ± 0.11[f]

Note: ATR = atrazine. GD = gestation day. PND = postnatal day.
Scoring:1 = stunted growth pattern; 4 = normal growth for age.
Dam $n > 8$,with ≥ 5 offspring per exposure group at each time.
All significant effects versus Control. Data presented as dam mean ± SE.
[a]Significant treatment effect by ANOVA (LSM); $p < 0.0005$.
[b]Significant treatment effect by ANOVA (LSM); $p < 0.0001$.
[c]Significant treatment effect by ANOVA (LSM); $p < 0.009$.
[d]Significant treatment effect by ANOVA (LSM); $p < 0.0495$.
[e]Significant treatment effect by ANOVA (LSM); $p < 0.0035$.
[f]Significant treatment effect by ANOVA (LSM); $p < 0.0004$.
[g]Significant treatment effect by ANOVA (LSM); $p < 0.0002$.
[h]Significant treatment effect by ANOVA (LSM); $p < 0.0287$.
[i]Significant treatment effect by ANOVA (LSM); $p < 0.0470$.
[j]Significant treatment effect by ANOVA (LSM); $p < 0.0119$.

By the peripubertal time point, PND 33, none of the ATR-exposed offspring glands were statistically smaller in area than controls (Table 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 (Fig. 3). Even though glands from exposed offspring were not smaller in area, the glands received lower developmental scores than controls. Atrazine-exposed glands displayed abundant terminal end buds on 2–3 sides of the epithelial tree, limited lobules, and sparse branching (Table 4). Figure 3A shows the distance between the fourth and fifth glands at PND 33, in addition to terminal end buds.

At early adulthood (PND 46), glands of ATR-exposed offspring were similar in length to controls (Table 3). The 4th and 5th 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 MG 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 MG was very dense with epithelial branching (Fig. 3C). 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

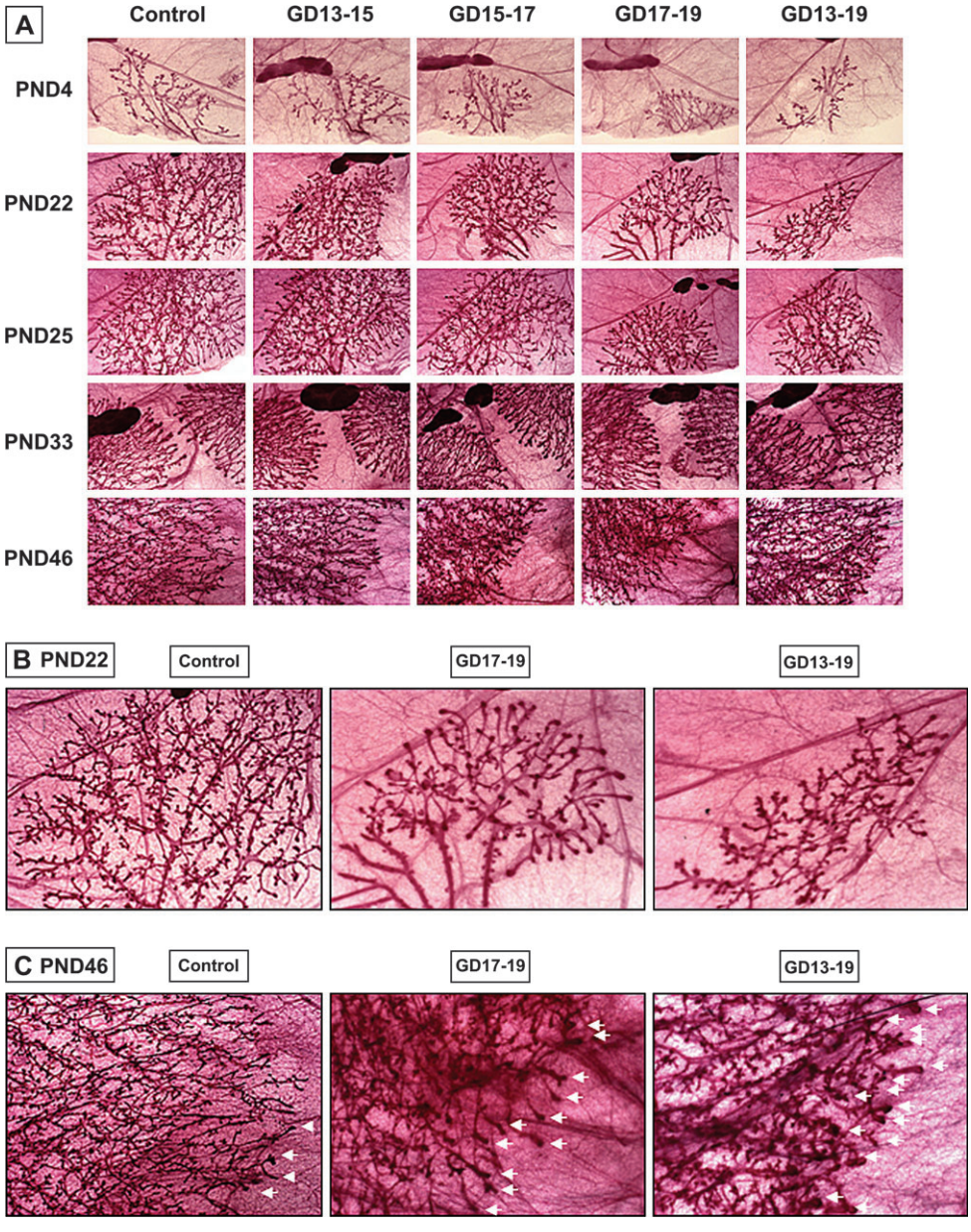

FIG. 3. Critical window for effects of prenatal exposure to 100 mg atrazine/kg BW/day on mammary gland (MG) development. (A) Mammary gland whole mounts were prepared from female offspring on postnatal days (PND) 4, 22, 25, 33, and 46 after ATR exposure on gestational days (GD) 13–15, 15–17, 17–19, or 13–19. Photos demonstrate the mean scores shown in Table 4. (B) Enlarged picture of PND 22 MG from control, GD 17–19, and GD 13–19, demonstrating fewer branches, delayed migration through the fat pad, and sparse appearance of atrazine-exposed glands. (C) Enlarged picture of PND 46 MG from control, GD 17–19, and GD 13–19. Glands taken from GD 17–19 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 (GD 13–19).

in GD 17–19 and GD 13–19 (arrows in Fig. 3C), leading to consistently low developmental scores (Table 4). At a later point in adulthood, when all females should have been sexually mature (PND 67), 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 GD 17–19 and GD 13–19 groups received epithelial development scores that were statistically equivalent over time (PND 4–67; Table 4). Control glands had few complex lobules and little dense branching throughout the gland, and they remained in the resting state described (not shown).

Serum hormone measurements. To determine if ATR exposure affected circulating hormone levels, serum was separated from trunk blood of animals at PND 33 and PND 67. At PND 33, corticosterone concentrations were significantly increased in the GD 15–17 group, $p < 0.009$;

TABLE 5
Lack of Effect of 100 mg/kg ATR on Reproductive Organ Weights and Serum Hormone Concentration (/ml) at PND 67

	Control	GD 13–15	GD 15–17	GD 17–19	GD 13–19
Body weight (g)	273.0 ± 3.5	261.1 ± 10.0	235.1 ± 5.3[a]	256.6 ± 5.0	243.8 ± 5.7[a]
Ovaries (mg)	115.9 ± 8.4	148.7 ± 10.2	146.0 ± 15.2	161.0 ± 20.8	132.5 ± 4.7
Uterus wet wt (mg)	310.1 ± 31.4	370.4 ± 35.5	430.2 ± 56.8	489.1 ± 112	418.4 ± 35.6
Progesterone (ng)	8.8 ± 2.7	7.6 ± 2.22	5.4 ± 2.5	11.6 ± 7.84	6.5 ± 1.6
Corticosterone (ng)	128.2 ± 17.4	118.7 ± 31.3	106.6 ± 11.5	112.7 ± 41.6	127.7 ± 22.2
Androstenedione (ng)	0.602 ± 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 ± 13.7	54.8 ± 5.0
Testosterone (ng)	0.16 ± 0.04	0.19 ± 0.07	0.2 ± 0.1	0.15 ± 0.09	0.29 ± 0.05
Estradiol (pg)	51.7 ± 2.3	46.6 ± 3.9	70.7 ± 33.4	59.5 ± 15.2	56.3 ± 4.8
Prolactin (ng)	6.9 ± 2.9	3.6 ± 0.3	4.5 ± 2.1	9.4 ± 4.3	8.8 ± 4.9

Note: ATR = atrazine, GD = gestation day. Dam $n > 4$ (>2 pups/dam). Data presented at dam mean ± SE.
[a]Significant treatment effect by ANOVA (LSM); $p < 0.01$ versus control.

72.1 ± 26.2, C vs. 193.9 ± 21.8, GD 15–17. Although no stress events were noted, we realized that this large increase could be due to an unknown environmental stressor prior to/during necropsy. Total testosterone in GD 13–19 animals was doubled compared to controls, $p < 0.04$; 0.203 ± 0.06, C vs 0.419 ± 0.08, GD 13–19. No additional differences in hormone concentrations evaluated at PND 33 were found.

At PND 67 (Table 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 GD 17–19 and GD 13–19 offspring. Estradiol was slightly increased in GD 15–17, GD 17–19, and GD 13–19 offspring, whereas its precursor testosterone was nearly doubled in GD 13–19 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 PND 67 (1 day before siblings were bred). In addition to the previous data described, the ovaries and uterus were removed and weighed. As shown in Table 5, there were no statistical differences among the groups in these organ weights with respect to day of cycle, even though there was a 14.3–38.9% increase in total ovary weight in the ATR-exposed animals versus controls. There was also a 19.4–57.7% increase in wet uterine weight compared to controls in the ATR-exposed females.

Consequences of Brief Prenatal ATR Exposure on Second Generation

The offspring of control and ATR-treated dams were bred to control LE males (beginning PND 68) to determine if the females exposed gestationally to ATR, and exhibiting delayed MG development, would have difficulty sustaining their young. Of the 20 normal-cycling females chosen to breed (4/group), only one female in the GD 17–19 group did not conceive. One female in the GD 13–19 group conceived, delivered two pups, but dam and pups died before PND 4. On PND 4, 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 PND 11, dams were removed from their pups for 2 h during a lactational challenge as described.

Female pups in the groups GD 17–19 and GD 13–19 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 $p < 0.0001$, and had body weight reduction of 15.6% and 16.7% (Fig. 4A). Mammary glands were removed from the female pups at PND 4 and scored for development and the area of the gland measured. Whole mount analysis of the fourth and fifth MG at PND 4 showed that mammary epithelial development of pups taken from GD 17–19 and GD 13–19 dams had few ductal buds from lateral branches and were undersized compared to glands taken from other groups. Control, GD 13–15, and GD 15–17 offspring glands displayed small buds on primary branches and moderate branching within the gland, and received similar developmental scores. When MG scores were compared using body weight as a cofactor, glands from GD 17–19 and GD 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 GD 13–15 and GD 15–17 ATR-exposure group (male and female) were significantly greater than the control group. The development of MG 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 6). The anterior pituitary glands (source of prolactin and decreased weight from ATR exposure; Laws *et al.*, 2000) were

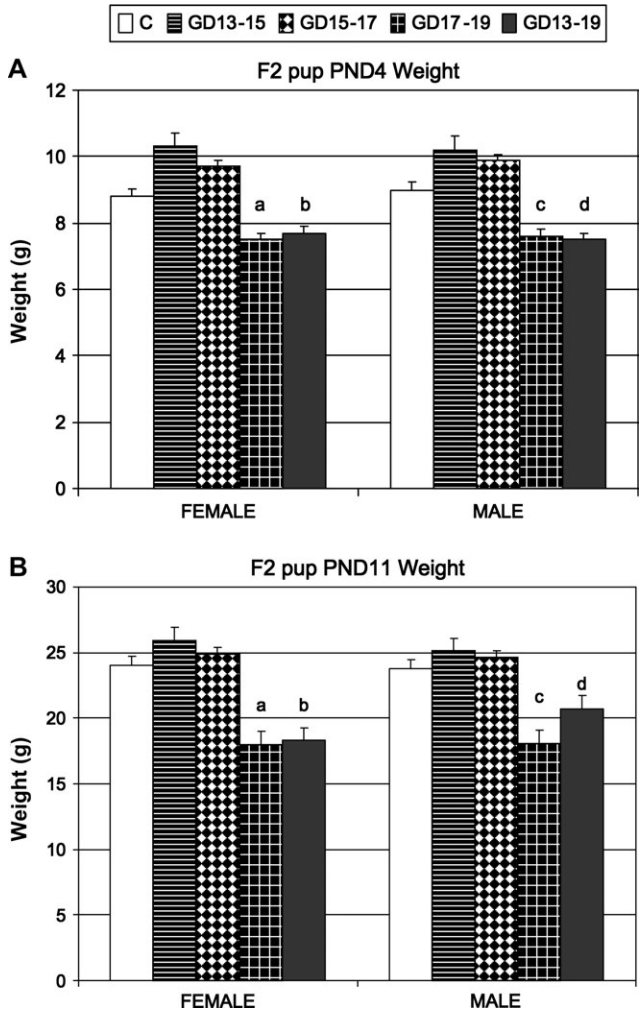

FIG. 4. Decreased mean body weight (BW, g) effects following gestational exposure to 100 mg atrazine/kg BW/day of second-generation pups (F_2) on PND 4 and PND 11. Data are presented separately for male and female pups as litter mean ± SE; $n > 3$ dams with >8 pups/dam (A) BW on PND 4. Significant treatment effect by ANOVA (LSM) and reduction in BW; different from control a = $p < 0.003$ (14.8%); b = $p < 0.02$ (12.5%); c = $p < 0.002$ (15.6%); d = $p < 0.0001$ (16.7%). (B) BW on PND 11. Significant treatment effect by ANOVA (LSM) and reduction in BW; different from control a = $p < 0.0001$ (25%); b = $p < 0.0003$ (23.8%); c = $p < 0.0001$ (23.9%); d = $p < 0.002$ (13%).

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 PND 11. Lower concentrations of estradiol were found in GD 13–15 and GD 13–19 dams, $p < 0.0006$ and $p < 0.02$, respectively. No other differences were found in hormone concentration among the groups, even though some values were quite varied (*e.g.,* PRL GD 17–19).

Body weights of the pups were recorded at PND 11, and again during GD 17–19 and GD 13–19 had dramatically reduced weight in both sex groups; female $p < 0.0001$ (25%) and $p < 0.0003$ (23.8%), male $p < 0.0001$ (23.9%) and $p < 0.002$ (13%) (Fig. 4B). Mammary glands removed from females at PND 11 were scored and measured. Glands taken from GD 13–15, GD 17–19, and GD 13–19 scored significantly lower than control and GD 15–17 glands. The areas of glands from GD 17–19 and GD 13–19 were reduced. As noted above, the pups from GD 17–19 and GD 13–19 dams were smaller at PND 11. This led us to evaluate MG 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 MG size or developmental score. Delays in MG development were not evaluated past PND 11 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 3 days to 100 mg/kg ATR during late gestation caused delayed MG development in the female offspring. These results also suggest that the fetal MG may be most sensitive to the effects of ATR during the latter part of pregnancy, the time of epithelial outgrowth. The MG of offspring exposed during GD 17–19 took longer to develop into mature glands. This developmental deficit was observed as early as PND 4 and as late as PND 67, when the animals were sexually mature.

Our studies suggest that GD 17–19 may be the most important period of fetal MG development, showing sensitivity to ATR that was consistent with the 7-day exposure. Our previous work showed that a 5-day *in utero* exposure to ATR during GD 15–19 delayed MG development in the female offspring (Rayner *et al*., 2004). We reported that gestational exposure combined with milk consumption from an ATR-treated dam (ATR-ATR) led to more severely delayed MG development than those exposed only *in utero* or lactationally. In the present study, a 3-day *in utero* exposure, especially during GD 17–19, caused MG developmental delays similar to that found in the 5-day ATR-ATR exposed animals (Rayner *et al.*, 2004), and the GD 13–19 offspring (7-day exposed; this study), framing what we believe to be the narrowest sensitive period of MG 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 MG. Our previous study and data presented here demonstrate that F_1 offspring body weight at GD 20, PND 4, or thereafter was not a significant variable influencing MG developmental

TABLE 6
Effect of 100 mg/kg ATR on Lactational Challenge and Serum Hormone Concentration (/ml) at PND 11

	Control	GD 13–15	GD 15–17	GD 17–19	GD 13–19
Body weight (g)	393.1 ± 19.2	386.1 ± 16.1	401.2 ± 15.0	353.3 ± 15.9	390.7 ± 13.5
Pituitary (mg)	15.7 ± 0.7	12.5 ± 1.0	15.4 ± 2.0	13.7 ± 0.4	16.9 ± 2.4
Nesting time (s)	287.5 ± 111	637.6 ± 121	390.2 ± 71.1	297.3 ± 68.1	306.6 ± 60.6
Estradiol (pg)	49.1 ± 2.9	32.0 ± 1.4[a]	43.9 ± 6.3	41.9 ± 2.9	33.6 ± 2.3[b]
Progesterone (ng)	58.8 ± 15	62.9 ± 4.8	44.7 ± 10	42.9 ± 21	62.2 ± 2.4
Prolactin (ng)	141.0 ± 50	109.7 ± 27	144.5 ± 68	21.0 ± 7.3	106.8 ± 57

Note: ATR = atrazine, GD = gestation day. Dam $n > 3$.
Data presented as dam mean ± SE. All effect versus Control.
[a]Significant treatment effect by ANOVA (LSM); $p < 0.006$.
[b]Significant treatment effect by ANOVA (LSM); $p < 0.02$.

delays induced by ATR. The present fetal weight study showed that dams treated with 50 mg/kg or 100 mg/kg of ATR during GD 15–19 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 3-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 GD 13–19 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) MG development were apparent in groups exposed to ATR, even after 3 days. Further, as noted in Table 6, there was no significant weight difference in F_1 dams used in the breeding study. Importantly, however, those same animals with delayed MG development at breeding (to generate the F_2 offspring) raised pups that were significantly (from 12% to 25%) smaller than controls, suggesting that the GD 17–19 and GD 13–19 ATR-exposed dams were not able to produce the quality and/or quantity of milk necessary to sustain the body weight of their offspring.

Epidemiological studies concentrating on sexual maturation and growth have been conducted in populations exposed to endocrine-disrupting compounds. Pubertal development was assessed by Blanck *et al*. (2000) in girls exposed to polybrominated biphenyls either *in utero* or through breastfeeding from maternal ingestion. The exposure occurred in 1973 after accidental contamination of Michigan dairy and animal products. These investigators found that females exposed to estimated high levels ($\geq$7 parts per billion; ppb) prenatally and lactationally experienced menarche at an earlier age than females exposed to low levels who were breastfed or than females who were not breastfed. Breast development in these females was not affected by exposure. Blanck *et al.* (2002) also examined the growth of these females. They found no association between height and weight and polybrominated biphenyl exposure. They did find that mothers who had higher ($\geq$5 ppb) polychlorinated biphenyl exposure had female children who weighed less than those exposed to high levels of polybrominated biphenyls. Den Hond and co-workers (2002) assessed two suburban Belgian adolescent populations exposed to polychlorinated aromatic hydrocarbons (PCAHs) and one rural cohort. The exposure sources were industrial, and samples were taken from the adolescent participants (60% female). 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 that a higher serum concentration of dioxin-like compounds was associated with delayed breast development, without a change in age at menarche. These studies begin to suggest that endocrine disrupting compounds can disrupt puberty and MG development in divergent ways, not only in rodents but in the human population as well.

Although a relatively high dose of atrazine (100 mg/kg) is used in these studies, it is important to remember that each pregnant rat contains in 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 two mammary buds developing in the average pregnant woman. The actual amount of ATR that reaches the developing mammary buds is the subject of on-going research in our lab.

We do not know the mode of action of ATR for the developmental delays in the rat MG. There were no changes in puberty or hormones that could be associated with delayed MG development in our study. Only the offspring from dams dosed during GD 13–19 exhibited delayed VO, indicating again (also in Rayner *et al.* [2004]) that VO is not altered in a time/dose paradigm similar to ATR-induced MG development delays. Delayed VO seems to require longer ATR exposures than do 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 begin earlier in both African American and Caucasian populations and they also take longer to conclude. However, in these populations, menses occurs at an age similar to that seen 30 years ago (Chumlea *et al.*, 2003). Many theories exist concerning the effect of environment on changes in sexual maturity. In our 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 PND 33 did not persist to PND 67 and were not outside the biologically normal value ranges. This further indicates that serum hormone levels are not a direct causal factor in delaying MG development after ATR exposure. It is entirely possible that the effect of ATR is an early effect and that the programed growth pattern of the mammary epithelium was altered by the prenatal exposures.

In an unpublished study prepared by Ciba-Geigy and accepted by the EPA (Mainiero *et al.*, 1987), rats were exposed to ATR over two generations. Male and female rats were treated through diet with approximately 0.5, 5, and 50 mg/kg/day ATR prior to mating, and then females were further 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 the two sets of parents were weight reductions at the high dose, but in the offspring no treatment-related changes were noted in the reproductive parameters evaluated, including fertility, postnatal mortality, or developmental delays. Our study, over two generations, consisted of treating only F_0 pregnant dams for 3 or 7 days with ATR by oral gavage. Female offspring displayed persistent delayed MG development (an endpoint not evaluated in NTP-like two-generational studies), but they appeared to have no changes in fertility or litter size. It was noted that F_2 litters in the GD 17–19 and GD 13–19 ATR-exposure groups were significantly reduced in body weight at both PND 4 and PND 11.

The mammary glands of these F_2 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. We observed delayed MG development in the siblings of the females bred on PND 68. It is possible that we would not have seen any F_2 effects if the females had been bred at a later time, such as at 90–120 days, as is typical in two-generation studies. We have no proof that this effect is permanent, just long-lasting.

In conclusion, MG developmental delays observed in all groups of ATR-exposed pups suggest that GD 17–19 is a sensitive window for fetal mammary gland development. While traditional endpoints were measured (body weight, puberty, and serum hormone concentrations), they were not associated with MG development effects in LE rats. Vorderstrasse *et al.* (2004) recently reported that mice exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin during pregnancy had trouble sustaining their young, resulting in pup death shortly after birth. Dioxin decreased branching, lobulo-alveolar development, and gland weight of treated dams when compared to controls. Fenton *et al.* (2002) demonstrated that acute exposure to dioxin on GD 15 in LE rats led to persistent delays in female offspring MG development. The MG development observed in dioxin-exposed offspring is similar to what we observed in the present study. Given this similarity, it would be interesting to determine if ATR has an adverse effect on MG differentiation of the exposed dam. Studies to determine if ATR impairs maternal MG development during pregnancy and if ATR has a direct effect on MG development in the fetus would certainly shed light on the mechanism by which ATR causes these early and fairly persistent effects on MG of rats.

DISCLAIMER

The information in this document was funded by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. Portions of the data within this manuscript were presented at the Society of Toxicology Meeting in Baltimore, MD, March 2004, and the Gordon Research Conferences Environmental Endocrine Disruptors meeting in New London, NH, June 2004.

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