

*Research Article*

# The Effect of Atrazine Administered by Gavage or in Diet on the LH Surge and Reproductive Performance in Intact Female Sprague-Dawley and Long Evans Rats

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Atrazine (ATR) blunts the hormone-induced luteinizing hormone (LH) surge, when administered by gavage (50–100 mg/kg/day for 4 days), in ovariectomized rats. In this study, we determined if comparable doses delivered either by gavage (bolus dose) or distributed in diet would reduce the LH surge and subsequently affect fertility in the intact female rat. ATR was administered daily to intact female Sprague-Dawley (SD) or Long Evans (LE) rats by gavage (0, 0.75, 1.5, 3, 6, 10, 12, 50, or 100 mg/kg/day) or diet (0, 30, 100, 160, 500, 660, or 1460 ppm) during one complete 4-day estrous cycle, starting on day of estrus. Estrous status, corpora lutea, ova, and LH plasma concentrations were evaluated. A second cohort of animals was mated on the fourth treatment day. Fertility metrics were assessed on gestational day 20. A higher portion of LE rats had asynchronous estrous cycles when compared to SD rats both during pretreatment and in response to ATR ( $\geq 50$  mg/kg). In contrast, bolus doses of ATR ( $\geq 50$  mg/kg) inhibited the peak and area under the curve for the preovulatory LH surge in SD but not LE animals. Likewise, only bolus-treated SD, not LE, rats displayed reduced mean number of corpora lutea and ova. There were no effects of ATR administered by gavage on mating, gravid number, or fetus number. Dietary administration had no effect on any reproductive parameter measured. These findings indicate that short duration, high-bolus doses of ATR can inhibit the LH surge and reduce the number of follicles ovulated; however, dietary administration has no effect on any endocrine or reproductive outcomes. *Birth Defects Res (Part B)* 101:262–275, 2014. © 2014 Wiley Periodicals, Inc.

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## INTRODUCTION

Atrazine (ATR) is a preemergence/early postemergence herbicide commonly used for weed control in corn, sorghum, and sugar cane (Breckenridge et al., 2010). ATR selectively inhibits electron transport systems in plant photosynthesis by reversible, competitive binding to an electron carrier substrate (Good, 1961; Tischer and Strotmann, 1977). When administered to rats, ATR has been shown to inhibit both the luteinizing hormone (LH) surge and pulsatile LH release (Cooper et al., 2000; Foradori et al., 2009a, 2009b). High doses of ATR have been shown to prolong the estrous cycle in aged, female Sprague-Dawley (SD) rats (Eldridge et al., 1994; Wetzel et al., 1994), delay the onset of puberty in both sexes (Stoker et al., 2002; Laws et al., 2003), and induce characteristics of premature reproductive aging in female SD rats (Eldridge et al., 1999a, 1999b). Furthermore, high

doses of ATR cause a reduction in LH and gonadotropin-releasing hormone (GnRH) pulse frequency (Foradori et al., 2009b, 2013). ATR reduces the degree of GnRH neuronal activation accompanying the reduction in the LH surge without reducing pituitary sensitivity to GnRH receptor activation (Foradori et al., 2009a, 2009b). These effects occur in the absence of any effect of ATR on the number of neurons expressing GnRH mRNA or changes in GnRH protein levels (Foradori et al., 2009a, 2013).

Additional Supporting Information may be found in the online version of this article.

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The majority of the work showing that ATR inhibits GnRH and LH release has been conducted using estrogen or estrogen plus progesterone primed ovariectomized rats. Hormone priming of ovariectomized animals allowed for the assessment of ATR effects on robust, reproducible, light-cycle entrained LH surges. However, this model cannot be used to evaluate the reproductive consequences of the effect of ATR on the LH surge. In addition, ovariectomy results in the loss of endogenous inhibitory feedback onto GnRH neurons and LH release, leading to an upward drive in output, which may mask possible, more subtle, inhibitory effects of ATR. Therefore in the present study, ATR was administered to intact female SD or Long Evans (LE) rats that had been prescreened for estrous cyclicity. The intact animal model was used to determine if ATR suppresses the endogenous preovulatory LH surge, and if so, whether similar treatment would result in reduced fertility as evidenced by mean reductions in number of oocytes ovulated, fetuses, and fertility indexes.

Previously, the effects of ATR on the hormone-induced LH surge have been characterized only following gavage (bolus) dosing, or in feeding studies in reproductively aged female SD rats (Simpkins et al., 2011). Since it is known that ATR is rapidly metabolized and cleared from plasma (McMullin et al., 2007), in the present study, the effect of dietary administration of ATR on the LH surge was also evaluated. The concentrations of ATR administered in feed were chosen to approximate the 24-h daily dose equivalent of the gavage dose that was effective in inhibiting the LH surge. Hence, the equivalent daily dietary administration of ATR was distributed across the day based upon the pattern of food intake and body weight during a pretest period. By examining the effect of bolus and distributed doses of ATR on multiple measures of reproductive outcome, the threshold ATR dose needed to suppress the LH surge and/or inhibit ovulation was determined for two strains of rats that have been previously shown to be sensitive to ATR (Cooper et al., 1996, 2000; Cummings et al., 2000; Narotsky et al., 2001). Furthermore, the relationship between the effects of ATR on the LH surge and the biology of reproduction in intact, normally cycling, female rats was characterized and compared to results obtained in traditional reproduction studies that were conducted by administering ATR in diet (DeSesso et al., *accepted-companion article*).

## METHODS

### Animals

All animal experimental protocols were approved by the Animal Care and Use Committee of WIL Research, Ashland, OH, where the in-life phase of the study was conducted, in accordance with NIH and AAALAC guidelines. Young adult female (60- to 90-day-old) SD and LE rats were purchased from Charles River Laboratories (Raleigh, NC). All animals were fed Certified Rodent LabDiet 5002 (PMI Nutrition International) with *ad libitum* access to water. Animals were housed individually in suspended wire-mesh cages and maintained on a 14-hr light:10-hr dark photoperiod under controlled temperature ( $22 \pm 3^\circ\text{C}$ ) and humidity ( $50 \pm 20\%$ ). Vaginal lavages were performed daily to determine the stage of the es-

trous cycle beginning at least 2 weeks prior to the first day of treatment. Only females displaying regular, 4-day estrous cycles for at least two consecutive cycles during the pretest period were used.

### ATR Treatment

ATR (certified to be 97.5% pure), which was supplied by Syngenta Crop Protection, LLC, was administered continually in the diet (distributed dose) or daily for 4 days by gavage (bolus dose) to intact female SD or LE rats. Only animals that displayed 4-day estrous cycles during the pretest period were included. Beginning a minimum of 7 days before the first dose, all animals were acclimated to treatment by administering daily doses of the gavage vehicle (1% methylcellulose in deionized water) at a volume of 5 ml/kg. Upon random assignment into groups, only those animals assigned to gavage treatment groups continued to receive the vehicle daily by gavage. Animals assigned to the dietary groups received control diet. Treatment was initiated on the day of estrus for animals in both the dietary and gavage groups and continued for four consecutive days.

Animals received daily oral gavage doses of ATR of 0, 0.75, 1.5, 3, 6, 10, 12, 50, or 100 mg/kg/day for SD and 0, 1.5, 3, 6, 12, 50 or 100 mg/kg/day for LE females at the time of lights on (05:00 hr). Control animals received the vehicle. A second cohort of animals received ATR-fortified rodent diet on a continuous basis beginning at 19:00 hr on the 4th day of the previous estrous cycle (estrus) and continuing over the next 4-day estrous cycle. ATR levels in diet were 30, 100, or 500 ppm for SD females and 160, 660, or 1460 for LE females. Control animals were given *ad libitum* access to control diet. Animals in the dietary groups were given either control diet or ATR-fortified diets on the evening of the 4th day of the prior estrous cycle and continuously over one 4-day estrous cycle. Diets containing ATR were prepared by weighing ATR into tared, glass mortars for each dosage group. ATR was ground with a small portion of rodent feed. The premix was transferred into a Hobart mixer with a total of 1 kg of rodent feed (weight/weight). A portion of the rodent feed was added to the glass mortar, which was then scraped into the Hobart mixer to ensure complete transfer of ATR. The formulation was mixed for 5 min. The remainder of rodent feed was weighed and placed in a V-blender, and the premix was then added to the blender to achieve the desired concentration. The diet was blended for 10 min (using an intensifier bar during the first and last 3 min). The homogeneity and stability of ATR in rodent feed was confirmed by HPLC before study conduct. For each batch of diet prepared, the concentration of ATR in the diet was verified before use and found to be within 90 to 110% of the targeted ATR concentration for each group.

The ATR and control diets were prepared weekly and stored at room temperature. A separate batch of diet was prepared for each dose group. The initial concentrations were based on average food consumption and body weight (BW) data collected during the pretest period. Dietary concentrations of ATR were adjusted as necessary throughout the study and were based on the mean BW and food consumption values for each group.

Table 1  
ATR's Effects on Estrous Cyclicity

SD bolus	Dose level (mg/kg/day)								
	0	0.75	1.5	3	6	10	12	50	100
Total no. of females evaluated	43	21	21	21	21	21	19	38	20
No. of females maintaining 4-day estrous cycles	39	18	20	19	20	20	18	27	15
No. of females displaying 5-day estrous cycles	2	1	1	1	1	1	1	8	3
No. of females that did not display estrus	2	2	0	1	0	0	0	3	2
Total no. (%) of females with irregular/abnormal cycles	4(9)	3(14)	1(5)	2(10)	1(5)	1(5)	1(5)	11(29)	5(25)
LE bolus	0	1.5	3	6	12	50	100		
Total no. of females evaluated	11	15	12	13	13	13	11		
No. of females maintaining 4-day estrous cycles	8	11	8	9	8	7	3		
No. of females displaying 5-day estrous cycles	0	2	3	3	1	4	4		
No. of females that did not display estrus	3	2	1	1	4	2	4		
Total no. (%) of females with irregular/abnormal cycles	3(27)	4(27)	4(33)	4(31)	5(38)	6(46)	8(73)		
SD distributed	Dietary concentration (ppm)								
	0	30	100	500					
Total no. of females evaluated	21	20	21	-	21	-	-		
No. of females maintaining 4-day estrous cycles	19	18	18	-	21	-	-		
No. of females displaying 5-day estrous cycles	0	1	1	-	0	-	-		
No. of females that did not display estrus	2	1	2	-	0	-	-		
Total no. (%) of females with irregular/abnormal cycles	2(9.5)	2(10)	3(14.3)	-	0(0)	-	-		
LE distributed	0	160	660	1460					
Total no. of females evaluated	11	-	-	12	-	11	14		
No. of females maintaining 4-day estrous cycles	11	-	-	12	-	9	13		
No. of females displaying 5-day estrous cycles	0	-	-	0	-	1	1		
No. of females that did not display estrus	0	-	-	0	-	1	0		
Total no. (%) of females with irregular/abnormal cycles	0(0)	-	-	0(0)	-	2(18)	1(7)		

### Experiment 1: Effect of Bolus Gavage or Dietary Distributed ATR Doses on the Estrous Cycle and Spontaneous LH Surge

ATR-fortified diet was offered on a continuous basis beginning at 19:00 hr on the 4th day of the previous estrous cycle (estrus) and was presented to rats over the next 4-day estrous cycle. ATR levels in diet were 30, 100, or 500 ppm for SD females and 160, 660, or 1460 for LE females. Control animals were given ad libitum access to control diet with 11 to 21 animals per treatment group (Table 1). A second cohort of animals received daily oral gavage doses of ATR at 0, 0.75, 1.5, 3, 6, 10, 12, 50, or 100 mg/kg/day for SD and 0, 1.5, 3, 6, 12, 50, or 100 mg/kg/day for LE females at the time of lights on (05:00 hr). Control animals received the vehicle with 11 to 43 animals per treatment group (Table 1).

Vaginal lavages were performed daily just after lights on during the pretreatment and treatment periods. On the last day of treatment, 250  $\mu$ l of blood was collected in prechilled heparinized tubes from each animal via jugular vein puncture at 6, 11, 13, and 18 hr post lights on. The 18:00 hr sample (4 hr post lights out) was collected under red light. All samples were centrifuged at 3000 rpm for 10 min at 4°C. Plasma samples were frozen, shipped on dry ice to the University of Arizona, College of Medicine, Phoenix, AZ, and stored at -70°C until analyzed by radioimmunoassay (RIA) to determine the plasma LH concentration.

Because each female was expected to begin a new estrous cycle the morning after the LH blood sample collection, a vaginal smear was collected to confirm that the animal was in estrus. If a female was not in estrus the following morning, blood samples were discarded, treatment continued, and a second set of blood samples was collected that evening. Vaginal smears were again obtained the following morning and if the female displayed an estrus smear, it remained in the study. If the animal was not in estrus, the stage of estrus was recorded and the animal was excluded from subsequent analysis.

Following collection of blood samples and the last vaginal smear (on the morning following the last blood sample collection), each female was euthanized by carbon dioxide inhalation. The abdominal cavity was opened, and the uterus was carefully dissected, trimmed to retain the luminal fluid. Each "wet" uterus was weighed intact (with the luminal fluid), then opened longitudinally, and blotted with filter paper to remove the luminal fluid. The ampulla of each oviduct was removed, placed on a clean glass slide, and opened, allowing the eggs within to spill out into saline. The number of ova on the slide was counted. Ovaries were grossly examined, and the number of corpora lutea (CL) was recorded. The number of CL and ova was recorded for all treatment groups and their respective control groups except for SD females administered ATR in the diet; the ATR dietary groups in experiment 1 were a subset of animals from an experiment where only the LH surge was assessed.

### Radioimmunoassay (RIA)

Plasma LH concentrations were determined by RIA at the University of Arizona College of Medicine, Phoenix, using reagents provided by the National Hormone and Peptide Program. Rat LH-RP3 was used for constructing standard curves. Ovine LH, iodinated using the chloramine T method by the Colorado State University peptide assay core, was used as the tracer. Plasma samples (50  $\mu$ l) were incubated overnight at room temperature (RT) with antiserum (NIDDK-Anti-rLH-SII, diluted 1:300,000). Following incubation, iodinated ovine LH was added to each tube (approximately 10,000 cpm/tube) and incubated overnight at RT. Bound LH was separated from free LH by incubation with goat anti-rabbit  $\gamma$  globin (EMD Millipore, Billerica, MA, cat no. 539845; 1:1000) in a 5% polyethylene glycol solution. Bound  $^{125}$ I-LH was counted with a Packard Cobra II gamma counter. The intraassay and interassay coefficients of variation for LH assays were 8.2 and 10.6%, respectively.

### Experiment 2: Effect of Bolus or Distributed ATR Doses on Reproductive Performance

Intact female rats were administered four daily gavage doses of ATR at levels of 0, 12, 50, or 100 mg/kg/day (SD) or 0, 1.5, 3, 6, 12, 50, or 100 mg/kg/day (LE) to determine if an ATR-induced reduction in the spontaneous preovulatory LH surge would result in reduced fertility. A subgroup of LE females received ATR in the feed at dietary concentrations of 160, 660, or 1460 ppm. Dietary studies that evaluated the effects of ATR on fertility in SD rats were conducted and are reported elsewhere (DeSesso et al., *accepted-companion paper*). Control animals were provided ad libitum access to control diet or gavaged with vehicle with 16 to 21 animals per treatment group (Table 3). All animals received ATR or the vehicle over one complete 4-day estrous cycle commencing at 05:00 hr of day of estrus. Vaginal lavages were performed daily for the determination of estrous cycle beginning at least 14 days before the assignment of individual animals to the study. The start of dosing for each female was based on the stage of estrus. On the last day of the treatment period, females were paired with untreated intact young adult male rats of the same strain (Charles River Laboratories). Females were placed into the male's home cage in the evening. If evidence of mating (i.e., the presence of a vaginal plug) was noted the following morning, the female was returned to an individual cage and the day was designated as day 0 of gestation. If there was no evidence of mating, a vaginal lavage was taken and the stage of estrus was recorded. If the female was in estrus, the female was separated from the male without further opportunity for mating. If the female was not in estrus on the morning following the initial pairing, treatment continued for an additional day and the female was paired with a second male. In the absence of mating on the second day, the stage of the estrous cycle was recorded and regardless of estrous stage, the female was separated from the male without further opportunity for mating. Pregnant females were euthanized on gestation day 20. Gravid uterine weight and dam BW were recorded. The number of fetuses was counted, and individual fetuses were weighed and sexed. The uteri, placentae, and ovaries

were examined; the total number of implantation sites, CL, and the number of late resorptions were counted.

### Data Analysis

Daily food consumption and BW data were analyzed using two-way ANOVA with dosage and day of treatment as factors. Fertility measures (number of ova and CL) were analyzed using one-way ANOVA. LH data were analyzed using repeated measures two-way ANOVA (treatment  $\times$  time). Peak LH amplitude was determined for each animal and the group mean was calculated, independent of the time that the blood sample was taken. The LH AUC was calculated using GraphPad Prism 5 software for each animal based upon LH values at each sampling time point. For AUC determinations, baseline LH levels were calculated as the mean of LH values in the first and last sample. If LH values were missing for any time point, the animal was not used in the calculation of the mean AUC. One-way ANOVA was performed on peak LH and AUC values to determine if there was an effect of ATR treatment. When a statistically significant *F* statistic was obtained, a Bonferroni *post hoc* test was used to determine which treatment groups were different from the control group. The level of statistical significance was set at  $p \leq 0.05$ . All data are presented as mean  $\pm$  SEM. Since there was an *a priori* hypothesis that reduced follicle and ova count would be associated with effects of ATR on the preovulatory LH surge, these data were also analyzed by comparing the control group to ATR treatment groups using one-tailed Welch's *t*-test, which does not depend on the assumption that variances are equivalent between groups. For binomial fertility metrics (number of animals mated, number of animals with evidence of mating, etc.), the differences between the corresponding proportional outcomes were tested using a Chi-squared test or Fisher's exact test. Continuous fertility metrics (number of fetuses per litter, number of implantation losses, etc.) were evaluated using a Welch's *t*-test.

The dose-response relationship for peak LH and LH AUC in SD and LE rats administered ATR by gavage was evaluated using the United States Environmental Protection Agency (USEPA) benchmark dose (BMD) software (USEPA, 2012). The BMDs and BMDLs (lower limit of a one-sided 95% confidence interval on the BMD) were based on exponential model 5 (the most general exponential model in BMDS), with no assumption of homoscedasticity (i.e., no assumption of equal variances), and with a benchmark response (BMR) equal to a decrease of one control standard deviation below the control mean. A similar analysis was attempted for dietary administered ATR, but could not be completed because there was no response to treatment.

## RESULTS

### Experiment 1: Effect of Bolus Gavage or Dietary Distributed ATR Doses on the Estrous Cycle and Spontaneous LH Surge

**Daily doses.** The daily doses in SD rats administered ATR by gavage were 0.75, 1.5, 3, 6, 10, 12, 50, or 100 mg/kg/day and 1.5, 3, 6, 12, 50, or 100 mg/kg/day in LE rats. The average calculated daily ATR doses in the

dietary-fed SD rats were  $2.7 \pm 0.06$ ,  $8.6 \pm 0.14$ , and  $41.8 \pm 1.07$  mg/kg/day in the 30, 100, or 500 ppm groups, respectively. The corresponding mean daily ATR doses in dietary LE females were  $10.2 \pm 0.27$ ,  $30 \pm 1.1$ , and  $45.6 \pm 1.97$  mg/kg/day, in the 160, 660, or 1460 ppm groups, respectively.

**Clinical signs, BW, food consumption.** There were no effects of ATR on behavior, clinical symptoms, or survival in either SD or LE rats, irrespective of whether ATR was administered by gavage or in the diet. There was no interaction between ATR dose and the duration of treatment with respect to BW in SD bolus ( $F_{(24,861)} = 0.05$ ;  $p = 0.16$ ), LE bolus ( $F_{(18,335)} = 0.05$ ;  $p = 1.0$ ), SD diet ( $F_{(9,318)} = 0.05$ ;  $p = 1.0$ ), or LE diet ( $F_{(9,182)} = 0.09$ ;  $p = 1.0$ ) treated animals. When the duration of treatment was removed as a factor, there was no effect of ATR treatment on BW in bolus-treated SD rats ( $F_{(8,861)} = 0.05$ ;  $p = 0.80$ ). There was a treatment effect of ATR on LE bolus rats ( $F_{(6,335)} = 4.7$ ;  $p < 0.001$ ). However, *post hoc* analysis failed to discern any differences between groups. In dietary animals there was an effect of ATR treatment (SD diet,  $F_{(3,318)} = 13.7$ ;  $p < 0.0001$ ; LE diet,  $F_{(3,81)} = 3.5$ ;  $p < 0.05$ ). Mean BW of dietary-treated, 500 ppm SD animals was lower than controls on day 2 of treatment and BWs of 1460 ppm LE animals were lower on days 2, 3, and 4 of treatment (Supplemental Table 1).

There was an ATR effect on food consumption in bolus SD ( $F_{(8,861)} = 29.1$ ;  $p < 0.0001$ ), bolus LE ( $F_{(6,332)} = 22.6$ ;  $p < 0.0001$ ), dietary SD ( $F_{(3,315)} = 41.9$ ;  $p < 0.0001$ ), and dietary LE ( $F_{(3,182)} = 123.6$ ;  $p < 0.0001$ ; Supplemental Table 2). In diet-treated animals (both SD and LE), all animals administered  $\geq 500$  ppm of ATR had reduced consumption on all days of treatment. There was no treatment effect on food consumption in either rat strain at dietary concentrations  $\leq 160$  ppm. In bolus SD animals, food consumption was reduced in the 50 mg/kg group on days 1 and 2 of treatment and on all days of treatment for the 100 mg/kg group. Daily food consumption was reduced in bolus LE animals treated with 50 mg/kg on day 1 of treatment and 100 mg/kg animals on days 1, 2, and 3 of treatment. There was no ATR effect on food consumption in either rat strains at doses  $\leq 12$  mg/kg.

**Estrous cycles.** During the pretreatment periods of experiment 1 and 2, significantly more LE rats (29.8%) displayed irregular, non-4-day estrous cycles over two successive cycles than did SD rats (19.8%; Fisher's exact test,  $p = 0.0061$ ; Supplemental Fig. 1 and Supplemental Table 3). Cycle abnormality consisted of LE rats spending significantly longer periods (days) in estrus ( $\geq 2$  consecutive days in estrus; LE—32.1% vs. SD—23.2%;  $p = 0.0102$ ) and in diestrus ( $> 3$  consecutive days in diestrus; LE—29.8% vs. SD—16.8%;  $p < 0.0001$ ) compared to SD rats (Supplemental Table 3).

Of the 43 SD females assigned to the vehicle gavage treatment group in experiment 1, on the final day of treatment, 39 of the animals maintained a 4-day cycle. Two of the four remaining animals were in estrus the following day (5-day cycle). Approximately 9% (4/43) of gavage-treated SD rats had irregular estrous cycles after treatment with vehicle. Females with irregular or abnormal cycles displayed either 5-day cycles or did not display estrus during the examination period. SD rats administered ATR by gavage at doses of 50 or 100 mg/kg/day over 4 days had an increase in the incidence of abnormal estrous cy-

cles compared to controls. The proportion (percent) of SD females that displayed irregular or abnormal estrous cycles following treatment were 4 of 43 (9%), 3 of 21 (14%), 1 of 21 (5%), 2 of 21 (10%), 1 of 21 (5%), 1 of 21 (5%), 1 of 19 (5%), 11 of 38 (29%), and 5 of 20 (25%) for the 0, 0.75, 1.5, 3, 6, 10, 12, 50, and 100 mg/kg/day groups, respectively.

Gavage-treated LE groups appeared to be more sensitive to the effect of treatment with the proportion (percent) of females that had irregular or abnormal cycles were 3 of 11 (27%), 4 of 15 (27%), 4 of 12 (33%), 4 of 13 (31%), 5 of 13 (38%), 6 of 13 (46%), 8 of 11 (73%) in the 0, 1.5, 3, 6, 12, 50, and 100 mg/kg/day dose groups, respectively. The percent of abnormal or irregular estrous cycles in the high-dose group (73%) was increased compared to vehicle controls (27%) and to the pretest period (29.8%; Table 1 and Supplemental Table 3).

The proportion (percent) of SD females in the ATR dietary groups that displayed irregular or abnormal cycles were 2 of 21 (9.5%), 2 of 20 (10%), 3 of 21 (14%), 0 of 21 (0%) for the 0, 30, 100, and 500 ppm groups, respectively. Of the ATR-treated females that were not in estrus on day 4, one female each in the 30 and 100 ppm groups was in estrus on the second day. All of the females in the 500 ppm group maintained 4-day estrous cycles following ATR treatment (Table 1).

In dietary-treated LE rats, all females in the control group were in estrus on the day after the final day of treatment, indicating that these animals maintained 4-day estrous cycles following vehicle treatment. In the 660 and 1460 ppm groups, 9 of 11 (82%) and 13 of 14 (93%) females, respectively, maintained 4-day estrous cycles. Single females in each of the 660 and 1460 ppm groups displayed 5-day estrous cycles, and 1 female in the 660 ppm group did not display estrus during the observation period. All females in the 160 ppm group maintained 4-day estrous cycles (Table 1).

**LH surge.** SD females administered ATR by gavage at doses of 50 and 100 mg/kg/day had significantly reduced mean LH levels at 11 and 13 hr post lights on (Fig. 1A;  $F_{(24,820)} = 2.75$ ;  $p < 0.0001$ ) when compared to controls. LH levels in the 1.5, 3, or 12 mg/kg/day dose groups were not different from the control group at any sampling time point. For bolus-dosed, ATR-treated LE animals, there was no significant interaction between treatment dose and time ( $F_{(18,318)} = 0.78$ ;  $p = 0.72$ ). However, there was a reduction in LH levels in the 100 mg/kg treated animals at 11 hr post light on (Fig. 1B;  $F_{(3,318)} = 19.9$ ;  $p < 0.0001$ ). There was no effect of dietary ATR on LH levels at any sample time point in either SD (Fig. 1C;  $F_{(9,298)} = 0.67$ ;  $p = 0.74$ ) or LE animals (Fig. 1D;  $F_{(9,176)} = 0.7$ ;  $p = 0.74$ ) groups.

Peak LH levels were lower in bolus-treated, SD females (Fig. 2A;  $F_{(8,215)} = 5.3$ ;  $p < 0.0001$ ) at the 50 and 100 mg/kg/day dose levels compared to controls. Although peak LH levels were reduced in the bolus-dosed LE groups, these differences were not statistically significant (Fig. 2B;  $F_{(6,87)} = 1.0$ ;  $p = 0.43$ ). Administration of ATR in the diet had no effect on peak LH levels in any dose group for SD (Fig. 2C;  $F_{(3,78)} = 0.27$ ;  $p = 0.85$ ) or LE animals (Fig. 2D;  $F_{(3,48)} = 0.27$ ;  $p = 0.85$ ).

The LH AUC was lower in the ATR-treated bolus-dosed, 50 and 100 mg/kg/day, SD groups compared to vehicle controls (Fig. 3A;  $F_{(8,203)} = 3.9$ ;  $p < 0.005$ ).

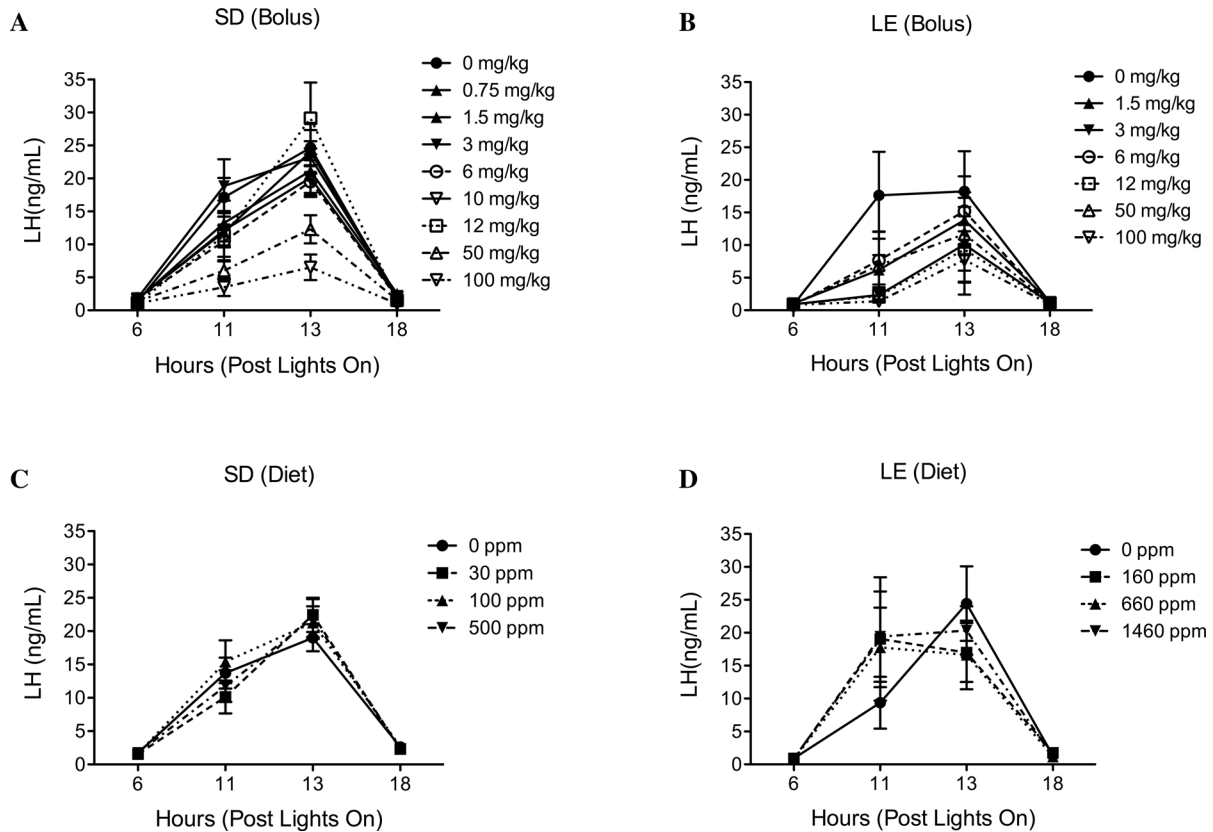


Fig. 1. Line graphs showing the mean  $\pm$  SEM LH levels of plasma on the evening of proestrus after 4 days of ATR treatment of intact female (A) SD bolus, (B) LE bolus, (C) SD dietary, or (D) LE dietary animals.

While lower, the reduction in LH AUC, in ATR-treated, bolus-dosed LE groups were not statistically significant (Fig. 3B;  $F_{(6,78)} = 0.7$ ;  $p = 0.64$ ). There was no difference in LH AUC in SD (Fig. 3C;  $F_{(3,74)} = 0.24$ ;  $p = 0.87$ ) or LE rats (Fig. 3D;  $F_{(3,43)} = 0.41$ ;  $p = 0.99$ ) administered ATR in the diet.

In SD rats administered ATR by gavage, the BMD and BMDL for peak LH were 53 and 50 mg/kg/day, respectively (USEPA, 2012). The corresponding values calculated based on LH AUC were 102 and 55 mg/kg/day, respectively. BMD and BMDL could not be calculated for SD distributed dosed groups or for LE bolus or distributed dose groups because no dose-response was evident (distributed dose) or because the response variance (LE bolus dose groups) was too large to model the data.

**Number of CL and ova.** Overall, a reduction in the mean number of CL per animal was observed in ATR-treated, bolus-dosed SD groups (Fig. 4, Table 2;  $F_{(3,78)} = 3.1$ ;  $p < 0.05$ ). The mean number of CL in the 100 mg/kg/day ATR group ( $10.7 \pm 1.7$ ) was significantly less than controls ( $15.4 \pm 0.6$ ). In addition, the mean number of ova shed per animal in both the 50 mg/kg/day ( $8.4 \pm 1.8$ ) and 100 mg/kg/day ( $8.1 \pm 1.9$ ) SD bolus dosed ATR groups were significantly less than controls ( $14.1 \pm 0.5$ ;  $F_{(3,78)} = 4.0$ ;  $p < 0.05$ ).

In LE rats, bolus ATR treatment did not result in a significant effect on the number of CL (Fig. 4; Table 2;  $F_{(6,87)} = 2.5$ ;  $p = 0.05$ ). The number of ova per animal was significantly reduced ( $F_{(6,87)} = 2.6$ ;  $p < 0.05$ ); however, *post*

*hoc* analyses did not reveal any group differences. Unpaired Welch's *t*-tests did identify significant differences between mean CL number per animal in the 3 mg/kg ( $7.8 \pm 2.0$ ;  $t(19) = 2.0$ ,  $p < 0.05$ ) and 100 mg/kg ( $6.3 \pm 1.9$ ;  $t(18) = 2.5$ ,  $p < 0.05$ ) groups compared to control animals ( $14.4 \pm 2.6$ ). Similarly, the unpaired Welch's *t*-test analysis showed that both 3 mg/kg ( $5.8 \pm 2.1$ ;  $t(20) = 2.0$ ,  $p < 0.05$ ) and 100 mg/kg ( $3.9 \pm 2.1$ ;  $t(19) = 2.4$ ,  $p < 0.05$ ) bolus-treated LE animals had significantly fewer ova compared to control animals ( $11.5 \pm 2.4$ ). There were no differences in CL ( $F_{(3,48)} = 1.8$ ;  $p = 0.2$ ) or ova ( $F_{(3,78)} = 1.0$ ;  $p = 0.4$ ) levels in LE groups administered ATR in their diet at any concentration.

To characterize the potential effect of ATR (bolus or distributed dose) on the mean number of CL or ova/animal, independent of the effect of ATR on the preovulatory LH surge, animals that failed to display a LH surge (arbitrarily defined as the failure of peak LH levels to be greater than twofold above baseline) were removed, and the ova and CL data were reanalyzed (Fig. 4, Table 2). Fifty-one of 54 animals removed for this subgroup analysis did not have any ova, indicating that these animals likely did not ovulate. When "nonsurging" animals were removed, there was no effect of ATR treatment on the mean number of CL in any treatment groups (Fig. 4; SD bolus,  $F_{(3,61)} = 1.5$ ;  $p = 0.2$ ; LE bolus, LE bolus,  $F_{(3,50)} = 0.7$ ;  $p = 0.7$ ; LE diet,  $F_{(3,44)} = 2.6$ ;  $p = 0.06$ ).

The mean number of ova identified in animals that were classified as producing a surge was found to be

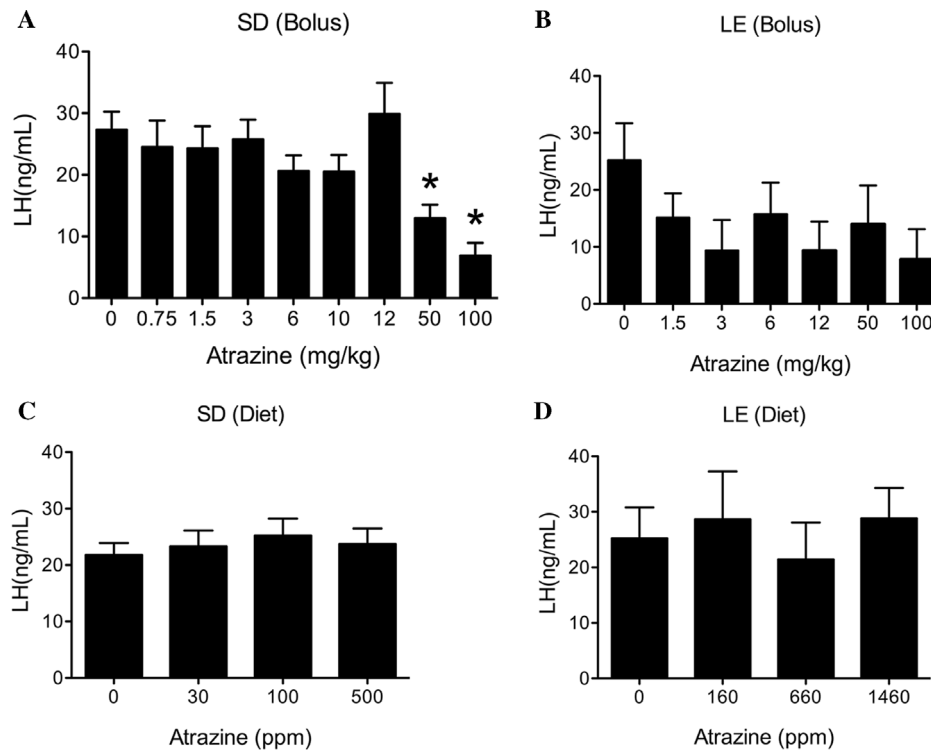


Fig. 2. Histograms showing the mean  $\pm$  SEM peaks of the LH surge after 4 days of ATR treatment of intact female (A) SD bolus, (B) LE bolus, (C) SD dietary, or (D) LE dietary animals. \*Significant difference ( $p < 0.05$ ) versus control group.

significantly different by one-way ANOVA for the SD bolus-treated animals ( $F_{(3,61)} = 2.8$ ;  $p < 0.05$ ). *Post hoc* analyses did not reveal any group differences, yet the unpaired Welch's *t*-test indicated that the mean ova number per animals in the SD bolus dosed, 100 mg/kg/day ( $16.1 \pm 1.0$ ) ATR group was significantly more than control "surging" animals ( $14.1 \pm 0.5$ ;  $t(28) = 1.8$ ,  $p < 0.05$ ). In "surging" LE animals, there were no effects of bolus ( $F_{(3,50)} = 0.6$ ;  $p = 0.7$ ) or dietary (Fig. 4, Table 2;  $F_{(3,44)} = 0.15$ ;  $p = 0.09$ ) administration on ova number.

### Experiment 2: Effect of Bolus or Distributed ATR Doses on Reproductive Performance

**Daily doses.** The daily doses in SD rats administered ATR by gavage were 12, 50, or 100 mg/kg/day and 1.5, 3, 6, 12, 50, or 100 mg/kg/day in LE rats. Mean equivalent daily ATR doses in the dietary groups of LE rats were  $11.2 \pm 0.3$  (160 ppm),  $35 \pm 0.8$  (660 ppm), and  $51 \pm 2.0$  (1460 ppm).

**Clinical signs, BW, food consumption.** As in experiment 1, there were no effects of ATR on behavior, clinical signs, or mortality in either SD or LE rats, irrespective of whether ATR was administered by gavage or in the diet (LE rats). There was no interaction between the ATR dose and the duration of treatment on BW. There was an effect of ATR treatment on BW in bolus SD animals ( $F_{(3,268)} = 8.0$ ;  $p < 0.0001$ ) with a difference on gestational day (GD) 0. There was no effect of ATR treatment on BW in bolus LE rats ( $F_{(6,516)} = 2.5$ ;  $p = 0.02$ ). In the dietary LE subgroup, mean BW was significantly

less than control on treatment day 2, 3, and 4 in the 1460 ppm group ( $F_{(3,268)} = 27.9$ ;  $p < 0.0001$ ; Supplemental Table 4).

Food consumption was reduced in the bolus SD-treated, 50 mg/kg group on day 2 and on all days in the 100 mg/kg treated animals ( $F_{(3,268)} = 43.5$ ;  $p < 0.0001$ ). Likewise, food consumption was reduced in bolus-treated, 100 mg/kg LE animals on all treatment days ( $F_{(6,508)} = 35.0$ ;  $p < 0.0001$ ). In dietary-treated LE groups, there was a significant interaction between treatment and duration of treatment for food consumption, which was reduced on every day of treatment in the 660 and 1460 ppm groups compared to controls ( $F_{(9,263)} = 5.44$ ;  $p < 0.0001$ ; Supplemental Table 5). There was a treatment effect on gestational BW in bolus-treated SD animals ( $F_{(3,432)} = 12.19$ ;  $p < 0.0001$ ; Supplemental Table 6); the 100 mg/kg group had a lower BW only on GD 0 compared to control (Supplemental Table 6). There was no effect of ATR treatment on BW in bolus LE animals ( $F_{(6,921)} = 1.5$ ;  $p = 0.2$ ). There was a treatment effect on gestational BW in the dietary LE animals ( $F_{(3,438)} = 11.0$ ;  $p < 0.0001$ ), with animals treated with 1460 ppm having a lower BW only on GD 0 compared to control (Supplemental Table 6).

During the gestation period, there was an effect of ATR on food consumption with bolus SD having higher intake on GD 0 but no other day ( $F_{(3,378)} = 3.6$ ;  $p < 0.05$ ; Supplemental Table 7). There was also a treatment effect in bolus LE-treated animals ( $F_{(6,805)} = 2.8$ ;  $p < 0.05$ ); however, *post hoc* analysis failed to discern a specific difference between doses on any day of gestation. There was no treatment effect on diet LE food intake at any dose or day ( $F_{(3,366)} = 1.0$ ;  $p = 0.37$ ).

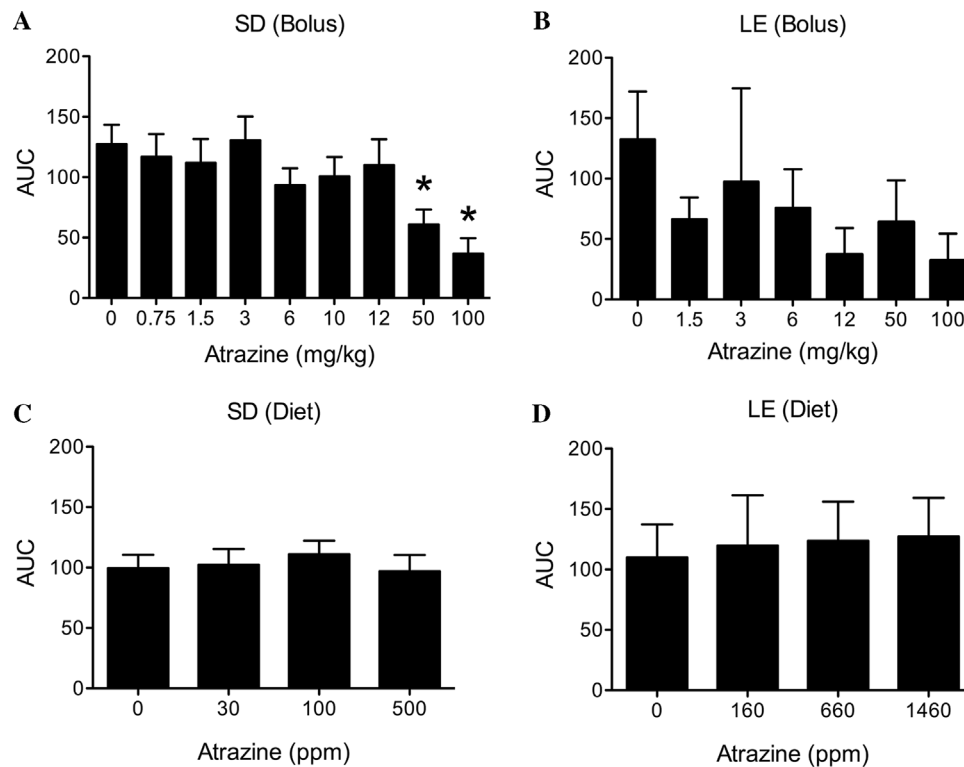


Fig. 3. Histograms showing the mean  $\pm$  SEM AUC of the LH surge after 4 days of ATR treatment of intact female (A) SD bolus, (B) LE bolus, (C) SD dietary, or (D) LE dietary animals. \*Significant difference ( $p < 0.05$ ) versus control group.

**Mating index, fertility index, postimplantation loss, fetal viability, and BW.** There were no effects of ATR treatment on the mating, fertility, or conception indices in SD or LE rats, regardless of dose or whether ATR was administered in diet (LE) or by gavage (Table 3). Likewise, there was no effect of ATR on the mean number of fetuses per litter at the termination of pregnancy. A significant increase in postimplantation loss in the 100 mg/kg bolus SD group ( $1.1 \pm 0.3$ ) was observed when compared to controls ( $0.4 \pm 0.1$ ;  $p < 0.05$ ; Table 3). There were no effects of treatment on fetal BW or sex ratio (data not shown).

## DISCUSSION

This study extends previous research showing that high doses of ATR administered by gavage inhibit the preovulatory surge (Cooper et al., 2007; Simpkins et al., 2011) by demonstrating that similar effects are found in normally cycling, intact young adult female SD rats. ATR administered as a daily bolus dose on each day of the 4-day estrous cycle significantly reduces peak LH and LH AUC in intact female SD rats at doses of 50 and 100 mg/kg/day, but not at doses  $\leq 12$  mg/kg. In contrast, bolus-treated LE rats did not have a statistically significant reduction in peak LH levels or AUC, but a reduction in LH levels in 100 mg/kg treated animals 11 hr post light on was observed. In a review article, Cooper et al. (2007) reported reduced peak LH plasma levels in intact LE rats administered bolus doses of 6.25, 12.5, and 25 mg/kg/day after treatment over a 4-day estrous cycle. Cooper et al. (2010),

in an internal EPA report, stated that ATR administered by gavage to LE rats at doses as low as 3.12 mg/kg caused a significant reduction in peak LH levels. Control LH levels in the Cooper et al. (2007, 2010) studies were comparable to those found in the present experiment, but variability was less. Calculated BMD and BMDL based upon peak LH or LH AUC in the present study were relatively uninformative because dose spacing between the no effect level of 12 mg/kg/day in bolus-dosed SD animals and the effect level of 50 mg/kg/day was not optimum. Furthermore large variances observed in LE bolus-dosed animals precluded calculating a BMD or BMDL.

In the current study, animals were bled over multiple time points to access individual LH surges, while Cooper et al. (2007, 2010) performed terminal bleeds to obtain single time point samples. Although Cooper et al. (2010) verified that animals treated over the estrous cycle were in proestrus (vaginal lavage and progesterone levels) on the day of blood sampling, the terminal bleeds precluded determination of estrus cytology the following morning. Animals failing to display estrus on the morning following blood sampling for the LH surge were removed from the current study. Thus, a subpopulation of LE rats, that may have been more sensitive to the effects of ATR, may have been inadvertently excluded from evaluation. However, the validation of estrus the following morning also led to a small portion of animals being found to not be in estrus and rebled the subsequent evening. Despite efforts to avoid stressing the animals, such a procedure could have led to increase stress for those animals and increased variability (Breen and Karsch, 2006). In addition, Cooper



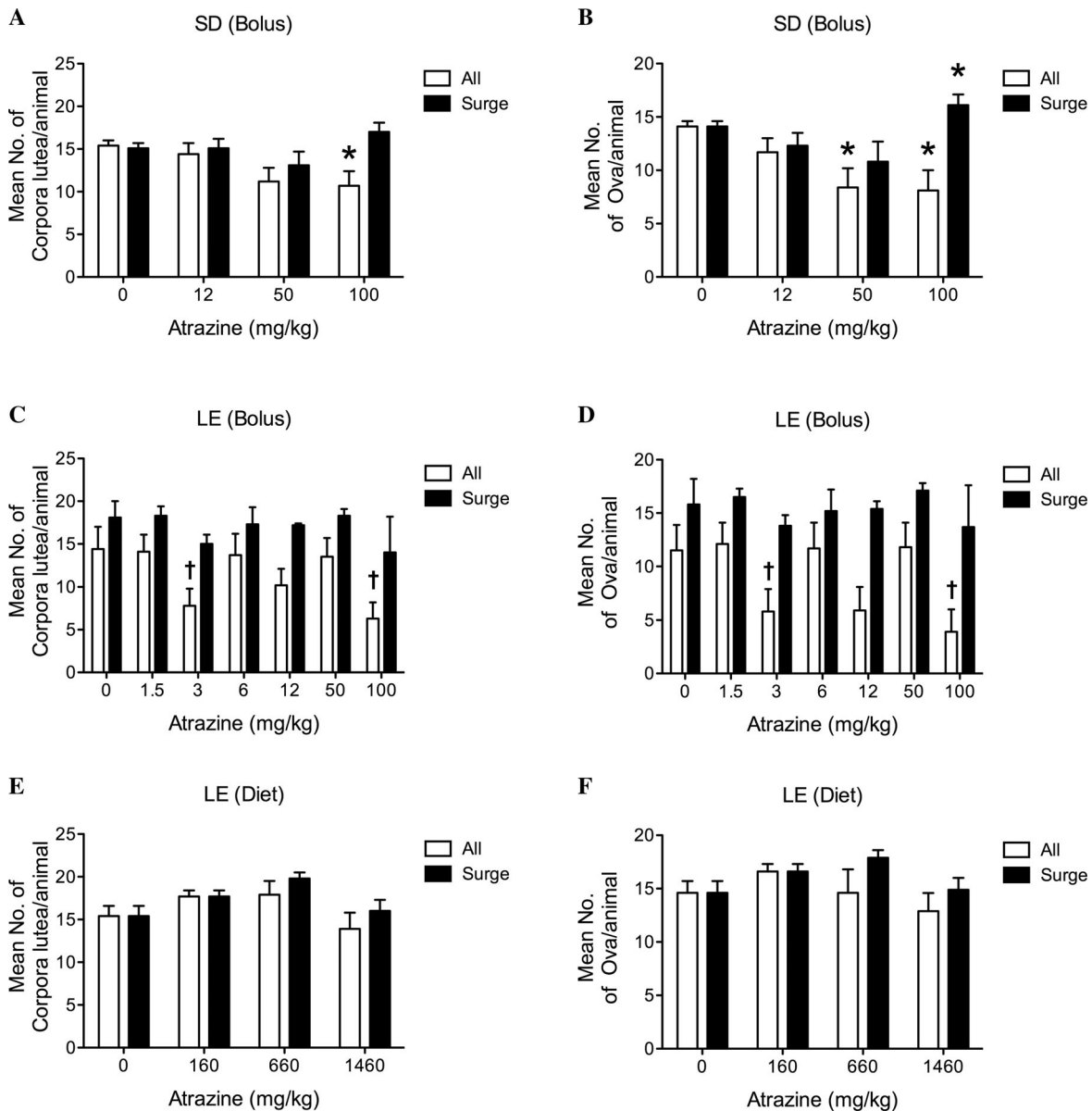


Fig. 4. Histograms showing the mean  $\pm$  SEM number of corpora lutea (A, C, E) and ova (B, D, F) after 4 days of ATR treatment of intact female SD bolus (A, B), LE bolus (C, D), and LE dietary (E, F) animals. \*Significant difference ( $p < 0.05$ ) versus control group using one-way ANOVA; †Significant difference ( $p < 0.05$ ) versus control group using Welch's *t*-test.

et al. (2007, 2010) gavaged animals at a time point 4 hr later than the present study. While the timing was not in the critical window/period of preovulatory LH surge disruption or delay (9–11 hr post lights on; Everett and Sawyer, 1949), it is clear from the present experiment that gavage administration of high doses of ATR in SD rats just after lights on for four successive days was sufficient to suppress the LH surge.

In contrast, when ATR was administered as a distributed dose in feed at daily equivalent doses averaging approximately 41.8 mg/kg/day in the SD and 45.6 mg/kg/day in LE rats, there were no effects of treatment on the LH surge. It may be that the dietary dose of ATR was not sufficient to suppress the LH surge. How-

ever, the dietary concentration of 500 ppm was selected for the SD rat because in a previous study 500 ppm was found to be the maximum tolerated dose (DeSesso et al., *accepted-companion paper*). Achieved daily doses of ATR in LE dietary groups were higher than in the dietary SD groups and were above effect levels reported by Cooper et al. (2010) in gavage-treated animals. ATR administered for 6 months in the diet at a concentration of 400 ppm has been shown to suppress the estrogen-induced LH surge in ovariectomized female SD rats (Simpkins et al., 2011). However, the animals in this study were older and hence approaching reproductive senescence, when the LH surge was assessed. As female SD rats approach middle age, the amplitude of the LH surge decreases (Cooper et al., 1980;

Table 2  
ATR's Effects on LH Surge and Ovulation

	Dose level (mg/kg/day)						
	0	1.5	3	6	12	50	100
SD bolus							
Total no. of females evaluated	21	-	-	-	20	18	20
Total no. of females displaying LH surge	19	-	-	-	19	14	10
Total mean no. of corpora lutea/animal	15.4 ± 0.6	-	-	-	14.4 ± 1.3	11.2 ± 1.6	10.7 ± 1.7*
Total mean no. of corpora lutea/animal displaying LH surge	15.1 ± 0.6	-	-	-	15.1 ± 1.1	13.1 ± 1.6	17.0 ± 1.1
Total mean no. of ova/animal	14.1 ± 0.5	-	-	-	11.7 ± 1.3	8.4 ± 1.8*	8.1 ± 1.9*
Total mean no. of ova/animal displaying LH surge	14.1 ± 0.5	-	-	-	12.3 ± 1.2	10.8 ± 1.9	16.1 ± 1.0*
LE bolus							
Total no. of females evaluated	11	15	12	13	13	13	11
Total no. of females displaying LH surge	8	11	5	10	5	9	3
Total mean no. of corpora lutea/animal	14.4 ± 2.6	14.1 ± 2.0	7.8 ± 2.0 <sup>†</sup>	13.7 ± 2.5	10.2 ± 1.9	13.5 ± 2.2	6.3 ± 1.9 <sup>†</sup>
Total mean no. of corpora lutea/animal displaying LH surge	18.1 ± 1.9	18.3 ± 1.1	15.0 ± 1.1	17.3 ± 2.0	17.2 ± 0.2	18.3 ± 0.8	14.0 ± 4.2
Total mean no. of ova/animal	11.5 ± 2.4	12.1 ± 2.0	5.8 ± 2.1 <sup>†</sup>	11.7 ± 2.4	5.9 ± 2.2	11.8 ± 2.3	3.9 ± 2.1 <sup>†</sup>
Total mean no. of ova/animal displaying LH surge	15.8 ± 1.3	16.5 ± 0.8	13.8 ± 1.0	15.2 ± 2.0	15.4 ± 0.7	17.1 ± 0.7	13.7 ± 3.9
	Dietary concentration (ppm)						
LE distributed	0			160		660	1460
Total no. of females evaluated	11	-	-	12	-	11	15
Total no. of females displaying LH surge	11	-	-	12	-	9	13
Total mean no. of corpora lutea/animal	15.4 ± 1.2	-	-	17.7 ± 0.7	-	17.9 ± 1.6	13.9 ± 1.9
Total mean no. of corpora lutea/animal displaying LH surge	15.4 ± 1.2	-	-	17.7 ± 0.7	-	19.8 ± 0.7	16.0 ± 1.3
Total mean no. of ova/animal	14.6 ± 1.1	-	-	16.6 ± 0.7	-	14.6 ± 2.2	12.9 ± 1.7
Total mean no. of ova/animal displaying LH surge	14.6 ± 1.1	-	-	16.6 ± 0.7	-	17.9 ± 0.7	14.9 ± 1.1

Data presented as mean ± SEM.

\* $p \leq 0.05$  when compared to control groups (0 ppm or 0 mg/kg) using one-way ANOVA with a Bonferroni post hoc.

<sup>†</sup> $p \leq 0.05$  when compared to control groups (0 ppm or 0 mg/kg) using Welch's *t*-test.

Wise, 1982; Simpkins et al., 2011). Although it is possible that the loss of GnRH drive on the pituitary may be sufficient to reveal a low-dose effect of ATR on the LH surge in older animals, it is recognized that reproductive aging in rodents is unlike and not relevant to humans (Neal-Perry and Santoro, 2006; Simpkins et al., 2011).

In the current study, high-dose ATR ( $\geq 50$  mg/kg) delivered via gavage for 4 days was able to reduce the preovulatory LH surge in gonadally intact SD animals. Previously, we and others have shown that hormone-induced LH surges in ovariectomized rats could be reduced in magnitude with four daily bolus doses of ATR (Cooper et al., 2000; McMullin et al., 2004; Foradori et al., 2011). Recently, Goldman et al. (2013) reported that four daily doses of ATR, administered by gavage, was required for ATR to inhibit LH surge. Interestingly, shorter treatment periods (after 1 dose) actually enhanced the LH surge, while 2 and 3 days of dosing had no effect (Goldman et al., 2013). The authors postulated that ATR works via the hypothalamic-pituitary-adrenal (HPA) axis to alter the LH surge. Gavage doses of ATR rapidly activate the HPA axis, as indicated by increased plasma concentrations of ACTH, corticosterone, and progesterone (Fraitas et al., 2009; Laws et al., 2009; Pruett et al.,

2009; Foradori et al., 2011). The mechanism whereby ATR induced activation of the HPA axis leads to an inhibition of the LH surge is not known. However, the time- and dose-dependent differential effect of ATR on LH surge may be due to a transition from progesterone as a positive to negative feedback on the HPG axis (Caligaris et al., 1971; DePaolo and Barraclough, 1979; Wagenmaker et al., 2009; Goldman et al., 2013).

While ATR-induced activation of the HPA axis needs further study, it is unlikely that the effect of ATR on the LH surge is mediated entirely through the HPA axis because adrenalectomy does not alter the effect of ATR on the LH surge (Foradori et al., 2011). Furthermore, gavage doses of diaminochlorotriazine (DACT), a metabolite of ATR, suppresses the LH surge, yet DACT does not have an effect on ACTH, corticosterone, or progesterone release (McMullin et al., 2004; Laws et al., 2009). Although the LH surge was reduced in ATR-treated, reproductively senescent, female SD rats (Simpkins et al., 2011), there is little evidence that long-term administration of ATR activates the HPA axis as indicated by the absence of an effect on immunological parameters that normally would be suppressed by glucocorticoids (unpublished data).

Table 3  
ATR's Effects on Fertility

	Dose level (mg/kg/day)						
	0	1.5	3	6	12	50	100
SD bolus							
Females paired for mating	18	-	-	-	17	17	18
No. of females with evidence of mating	16	-	-	-	16	12	15
No. gravid	16	-	-	-	15	12	15
Mean no. of fetuses	15.8 ± 0.5	-	-	-	15.1 ± 0.6	15.7 ± 1.3	16.2 ± 0.6
Mean no. of postimplantation loss	0.4 ± 0.1	-	-	-	0.7 ± 0.2	0.9 ± 0.4	1.1 ± 0.3 <sup>†</sup>
Fertility indexes							
Mating index	88.9	-	-	-	94.1	70.6	83.3
Fertility index	88.9	-	-	-	88.2	70.6	83.3
Conception index	100	-	-	-	93.7	100	100
LE bolus	0	1.5	3	6	12	50	100
Females paired for mating	20	19	21	18	20	18	19
No. of females with evidence of mating	19	19	20	17	18	16	18
No. gravid	19	18	20	16	18	16	16
Mean no. of fetuses	14.5 ± 0.9	14 ± 0.5	13.7 ± 0.8	14.8 ± 0.6	14.1 ± 0.9	15.3 ± 0.8	15.0 ± 0.6
Mean no. of postimplantation loss	0.9 ± 0.3	1.3 ± 0.4	0.7 ± 0.2	0.9 ± 0.2	0.8 ± 0.3	0.4 ± 0.2	1.0 ± 0.2
Fertility indexes							
Mating index	95.0	100	95.2	94.4	90.0	88.9	94.7
Fertility index	95.0	94.7	95.2	88.9	90.0	88.9	84.2
Conception index	100	94.7	100	94.1	100	100	88.9
	Dietary concentration (ppm)						
LE distributed	0	160	660	1460			
Females paired for mating	18	-	-	16	-	18	19
No. of females with evidence of mating	16	-	-	14	-	14	18
No. gravid	16	-	-	14	-	13	16
Mean no. of fetuses	14.4 ± 0.5	-	-	15.0 ± 0.5	-	14.8 ± 1.1	13.4 ± 1.0
Mean no. of postimplantation loss	0.8 ± 0.3	-	-	1.4 ± 0.4	-	1.5 ± 0.5	0.8 ± 0.2
Fertility indexes							
Mating index	88.9	-	-	87.5	-	77.8	94.7
Fertility index	88.9	-	-	87.5	-	72.2	84.2
Conception index	100	-	-	100	-	92.9	88.9

Mating index = (number of sperm-positive females/number of females cohabited with males) × 100.

Fertility index = (number of pregnant females/number of females cohabited with males) × 100.

Conception index = (number of gravid females/number of sperm-positive females) × 100.

<sup>†</sup>*p* ≤ 0.05 when compared to control groups (0 ppm or 0 mg/kg) using Welch's *t*-test.

*In vivo*, ATR is rapidly metabolized to the mono-dealkylated metabolites (de-ethylatrazine [DEA] and deisopropylatrazine [DIA]) via cytochrome P450 enzymes with subsequent conversion to the di-dealkylated metabolite, DACT (Hanioka et al., 1998; McMullin et al., 2003; McMullin et al., 2007). Dietary administration of ATR resulted in an approximate 10-fold lower peak plasma concentration of ATZ, DEA, and DIA and a twofold lower peak in DACT plasma concentrations compared to equivalent gavage doses (unpublished data). These differences in plasma concentration may explain in part why dietary administration of ATR had no effect on the LH surge, compared to the robust response seen in gavage-treated SD rats. It is likely that the chlorotriazines act at a site(s) in the brain upstream from the GnRH neuron (Foradori et al., 2013) and daily plasma concentrations of the chlorotriazines above a critical threshold are necessary to suppress the LH surge.

In an unpublished pharmacokinetic study, the time to peak concentration following bolus dose administration

of 50 mg/kg ATR was 0.5, 0.9, 1.3, and 8.0 hr for the ATR, DEA, DIA, and DACT, respectively, and the half-life of clearance from plasma was 2.4, 6.9, 6.0, and 8.1 hr, respectively (unpublished data). The corresponding time to peak concentration following 500 ppm dietary administration (43 mg/kg) was 16, 16, 16, and 18 hr for ATR, DEA, DIA, and DACT, respectively, and the half-life of clearance from plasma was 2.3, 7.0, 3.9, and 11.5 hr, respectively. Rapid pharmacokinetics and the low potential of the chlorotriazine metabolites to bioconcentrate in tissue (Solomon et al., 2008) further exacerbate the differences between gavage and dietary dosing. This interpretation is supported by the observation that 4 days of dosing is needed to suppress the LH surge (Goldman et al., 2013) and the LH surge returns to control levels 4 days after cessation of ATR treatment (Foradori et al., 2009a).

Previous studies have shown that short duration high-dose gavage studies (Peters and Cook, 1973; Cooper et al., 1996) and longer duration feeding studies on ATR (Wetzel et al., 1994) altered estrous cycles in female SD rats. Other

groups have shown that short duration high-bolus doses of ATR resulted in prolonged diestrus (Peters and Cook, 1973; Cooper et al., 1999) and long duration, lower doses resulted in persistent estrous (Wetzel et al., 1994). The present study confirms the results of previous work in that ATR doses  $\geq 50$  mg/kg administered by gavage to SD or LE rats resulted in an elevated percent of animals with abnormal estrous cycles. LE animals appear to be more sensitive to the effects of ATR on the estrous cycle than SD rats. The percentage of LE rats displaying irregular estrous cycles was 27, 46, and 73%, in the 0, 50, and 100 mg/kg/day dose groups, respectively, compared to 9, 29, and 25%, respectively, in the comparable SD groups. In addition, during the prescreening period to assure only regular 4-day cycling animals were used in the study, a significantly higher portion of LE animals displayed irregular cyclicity (29.8% of LE vs. 19.8% of SD). These results indicate that LE rats have more variable estrous cycles than SD rats, not only under normal conditions, but also exhibit a greater cycle disruption in response to short duration, high gavage doses of ATR. In contrast to the gavage-treated groups, dietary administration of ATR for 4 days at feeding levels up to 500 ppm (41.8 mg/kg/day) in SD rats and 1460 ppm (45.6 mg/kg/day) in LE females had no effect on the estrous cycle, which is likely explained by the rapid metabolism of ATR.

ATR administered by gavage at doses that suppressed the LH surge in female SD rats (i.e., doses of 50 and 100 mg/kg) also resulted in a significant reduction in the number of CL and ova shed. These results are consistent with those reported by Cooper et al. (1996). When animals, whose LH levels did not reach an arbitrarily defined LH level of twofold higher compared to baseline, were removed from the analysis, the mean ova and CL numbers were no longer significantly different from the control group. This suggests that ATR-associated reduction in ova was due to a reduced LH surge and not a direct effect on ovarian function.

The fact that the number of CL was only reduced in animals treated with 100 mg/kg may indicate that ATR at a dose of  $\geq 100$  mg/kg disrupts the estrous cycle and possibly follicle development along with the LH surge. The reduction in mean number of ova in SD females administered 50 mg/kg ATR by gavage, without a concomitant effect on the mean number of follicles (Fig. 2, Table 2) suggest that the 50 mg/kg bolus dose of ATR had no effect on follicular development, even though LH was suppressed (Wu et al., 1987; Wilson et al., 1995). This interpretation is consistent with Foradori et al. (2013), who showed GnRH pulse frequency was altered by high doses of ATR when administered by gavage. Although follicle recruitment had begun before the first ATR exposure, disruption of GnRH pulse frequency in some bolus-treated animals might have led to reduced follicle maturation (Peluso et al., 1984; Gong et al., 1996). In contrast, administration of ATR in the diet to SD or LE rats did not suppress the LH surge and did not have any effect on the number of CL or ova.

There was no effect of ATR treatment, administered by gavage or in the diet, on any measure of reproductive performance, including mating, fertility, and conception indices, or mean number of pups/litter (Table 3). The statistically significant increase in postimplantation

loss in the 100 mg/kg bolus-dosed SD females (1.1% compared to controls (0.4%) is unlikely to be related to treatment since Scialli et al. (*accepted-companion paper*) found no evidence of postimplantation loss at doses of 70 or 100 mg/kg although both pre- and postimplantation loss occurred at a dose of 700 mg/kg in two SD rats studies administering ATR from GD 6 to 15.

The results in the dietary subgroups are consistent with the lack of effect of ATR on fertility in multigeneration reproduction studies (DeSesso et al., *accepted-companion paper*). The absence of any effect of gavage doses of ATR on fertility or the number of fetuses is surprising given that the LH surge and ova count were reduced in groups of animals in experiment 1 that were euthanized the day after the occurrence of the preovulatory LH surge. It is possible that the animals allowed to mate on the evening of proestrus had a copulation-induced secondary LH surge that supported the ovulation of all follicles that had been recruited earlier in the estrous cycle. Copulation-induced LH surges have been previously reported in rats (Blake and Sawyer, 1972; Rajendren et al., 1993). Alternatively, ATR treatment could have delayed the LH surge for 24 hr, which is a common finding on female rats receiving various chemical treatments (Barracough and Sawyer, 1955; Baldwin and Sawyer, 1974; Li et al., 1995). However, commonly, these compounds were given on proestrus and did not result in a delay in estrus cytology, but extended the cycle with a second day of vaginal cornification. When these compounds were given earlier in the estrous cycle, again the cycle was prolonged with repeated days in diestrus (Baldwin and Sawyer, 1974). No such changes to the estrous cycles were seen in the present study. Similarly, delayed ovulation has been shown to be associated with increased implantation losses (Butcher et al., 1969) that were not observed in the present study regardless of dose delivery method or strain of animal (Table 3).

In summary, these studies show that, although in intact SD and LE rats high bolus doses of ATR significantly reduced the preovulatory LH surge and the number of CL and ova shed (SD), there were no ATR effects on fertility or litter size. ATR administered, as a distributed dose in diet to SD and LE rats did not have any effect on the LH surge, number of CL, or ova shed. Mating, conception and fertility indices as well as mean postimplantation loss and number of fetuses at the termination of pregnancy were comparable to controls.

Taken together, the results in this study suggest that the cellular mechanisms within the endocrine system responsible for fertility are robust and that marked reduction in the LH surge have little effect on overall reproductive performance in rodents. In addition, ATR effects on the rat LH surge are dependent on bolus delivery, most likely resulting from achieving critical plasma concentration of ATR and/or its chlorometabolites. Equivalent daily doses of ATR distributed over a 24-hr period had no effect likely because critical plasma and target tissue concentrations were not achieved. Thus, the effects of ATR on the LH surge and ovulation following bolus doses are highly unlikely to occur in humans exposed at low, temporally distributed, concentrations of ATR in drinking water. In conclusion, the regulatory standard established for exposure to the chlorotriazines (USEPA, 2006;

WHO, 2009; 2010) that are based upon the observed effect level (1.8 mg/kg/day) for LH surge suppression in reproductively aged, SD rats (Simpkins et al., 2011) adequately protect against any adverse reproductive effect occurring in humans.

### CONFLICT OF INTEREST

The studies summarized in this article were conducted by Syngenta Crop Protection, LLC, a manufacturer and registrant of atrazine. Drs. Breckenridge and Yi are employees of Syngenta and Merrill Tisdell is a former employee. Dr. Coder was employed by WIL Research where the in-life phase of the studies was conducted under contract to Syngenta. Drs. Foradori, Handa, and Simpkins are either consultants to Syngenta or conducted research described herein as part of research grants from Syngenta.

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