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Menstrual cycle characteristics and reproductive hormone levels in women exposed to atrazine in drinking water ☆

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ABSTRACT

Atrazine is the most commonly used herbicide in the U.S. and a wide-spread groundwater contaminant. Epidemiologic and laboratory evidence exists that atrazine disrupts reproductive health and hormone secretion. We examined the relationship between exposure to atrazine in drinking water and menstrual cycle function including reproductive hormone levels.

Women 18–40 years old residing in agricultural communities where atrazine is used extensively (Illinois) and sparingly (Vermont) answered a questionnaire ($n=102$), maintained menstrual cycle diaries ($n=67$), and provided daily urine samples for analyses of luteinizing hormone (LH), and estradiol and progesterone metabolites ($n=35$). Markers of exposures included state of residence, atrazine and chlorotriazine concentrations in tap water, municipal water and urine, and estimated dose from water consumption.

Women who lived in Illinois were more likely to report menstrual cycle length irregularity (odds ratio (OR)=4.69; 95% confidence interval (CI): 1.58–13.95) and more than 6 weeks between periods (OR=6.16; 95% CI: 1.29–29.38) than those who lived in Vermont. Consumption of >2 cups of unfiltered Illinois water daily was associated with increased risk of irregular periods (OR=5.73; 95% CI: 1.58–20.77). Estimated "dose" of atrazine and chlorotriazine from tap water was inversely related to mean mid-luteal estradiol metabolite. Atrazine "dose" from municipal concentrations was directly related to follicular phase length and inversely related to mean mid-luteal progesterone metabolite levels.

We present preliminary evidence that atrazine exposure, at levels below the US EPA MCL, is associated with increased menstrual cycle irregularity, longer follicular phases, and decreased levels of menstrual cycle endocrine biomarkers of infertile ovulatory cycles.

1. Introduction

Atrazine, a triazine herbicide applied to a variety of crops for weed control, is the most commonly used herbicide in the United States (U.S. Environmental Protection Agency, 2008) and a frequently detected contaminant in surface and drinking water in high use areas (US Environmental Protection Agency, 2003).

Concerns regarding potential health effects of exposure to atrazine in humans are based in part on reported adverse neuroendocrine and reproductive effects and the disruption of reproductive hormones including inhibition of luteinizing hormone (LH) release in laboratory animals (Cooper et al., 1996, 2007, 2000; Gojmerac et al., 2004; McMullin et al., 2004; Narotsky et al., 2001; Trentacoste et al., 2001).

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In toxicological studies, exposure to chlorinated triazines, which include atrazine and its metabolites has been shown to cause altered estrous cycles, delayed puberty, pregnancy loss, prostate inflammation, hermaphroditism and gonadal dysgenesis (Cooper et al., 2007; Hayes et al., 2002, 2003; Laws et al., 2000; Narotsky et al., 2001; Stevens et al., 1994; Stoker et al., 1999; Wetzel et al., 1994).

In humans, exposure to atrazine has been associated with intrauterine growth retardation (IUGR) (Munger et al., 1997), small-for-gestational-age (SGA) births (Ochoa-Acuna et al., 2009; Villanueva et al., 2005), spontaneous abortion (Arbuckle et al., 2001) and reduced semen quality (Swan, 2006). In the Agricultural Health Study, women who reported using pesticides, including atrazine, had an increased risk of missed periods and intermenstrual bleeding; those who mixed or applied atrazine or lindane reported longer menstrual cycles (Farr et al., 2004). Menstrual cycle characteristics, including the underlying endocrine axis, have implications not only as biomarkers of fertility issues (e.g., fecundity, spontaneous abortions, endometriosis, uterine fibroids), but are also associated with heightened risk to hormonally sensitive diseases (e.g., cancers, osteoporosis, cardiovascular disease and diabetes) (Charkoudian and Joyner, 2004; Deroo and Korach, 2006; Shuster et al., 2008, 2010; Xiao et al., 2006).

Thus, our goal was to explore the relationship between exposure to atrazine in drinking water and human menstrual cycle function, including menstrual cycle characteristics and associated hormone levels.

2. Materials and methods

2.1. Participant selection

The Illinois communities of Mount Olive and Gillespie had among the highest atrazine municipal drinking water concentrations in the nation in 2003 (Atrazine Monitoring Program, Syngenta Crop Protection, Inc.) and were selected for study as a high-exposure population. Raw water concentrations of atrazine and chlorotriazine in 2003, respectively, were 18.8 and 20.6 μg/L in Mount Olive and 5.1 and 7.2 μg/L in Gillespie. Waterbury and Fair Haven, Vermont, were selected as a low-exposure population. Vermont is an agricultural state where small amounts of atrazine are used.

The municipal offices of Mount Olive, Gillespie, Fair Haven and Waterbury provided contact information (name, address and phone number) for all residences served by their respective water utility. Each residence was sent a letter explaining the study design, data collection procedures and informed consent procedure. Subsequently, the investigator telephoned to determine whether an eligible woman lived at the residence and, if so, whether she was willing to participate. Potential participants were informed that the study focused on pesticides and reproductive health.

Participants were premenopausal women 18–40 years old residing in one of these communities in 2005. Women were not eligible to participate if they had taken any form of hormonal contraception, medication or replacement, used an intrauterine device, breast fed within the past 3 months or were pregnant within the past 6 months. Women who had been diagnosed with disorders known to affect reproduction or endocrine function were also ineligible. No eligible participants resided at 1022 (82.42%) of the homes called.

All participants ($n=102$) answered a questionnaire that included reproductive history, information on potential confounders, exposure indices and menstrual cycle characteristics. A subset of these women ($n=67$) maintained a daily diary of vaginal bleeding for one complete menstrual cycle and provided paired urine and home tap water samples on the same day. Thirty-five of these women also collected daily first-morning urine samples for a complete cycle. Each woman chose the extent of her participation. Informed consent was obtained following guidelines established by the Colorado State University Human Subjects Research Committee.

2.2. Exposure assessment

Markers of atrazine exposure, including state of residence, years in current home, and consumption of unfiltered water, were obtained from questionnaires. The daily volume of unfiltered tap water ingested, including drinks made with tap water, was calculated for each participant.

Two home tap water samples and two urine samples were collected two days apart to further assess exposure. Cold tap water was collected in pre-washed amber glass bottles with Teflon screen caps (US Environmental Protection Agency, 1994) after running the system for two minutes. Sodium sulfite (4–5 mg) was added to each water sample to remove residual chlorine (US Environmental Protection Agency, 1994). Urine samples were collected in prewashed bottles (US Environmental Protection Agency, 1994). Tap water and urine samples were placed on ice during transport to the field station, frozen at −20 °C and shipped on dry ice by next-day courier to the Centers for Disease Control and Prevention (CDC) for analysis.

Tap water and urine samples were analyzed for atrazine, the chlorotriazines desethylatrazine, desisopropyl atrazine, and diaminochlorotriazine and the atrazine metabolites atrazine mercapturate and desethylatrazine mercapturate. Samples were analyzed using online solid phase extraction coupled with high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Panuwet et al., 2008). The limit of detection (LOD) was 0.5 μg/L for atrazine, atrazine mercapturate, and desethylatrazine mercapturate and 1.0 μg/L for desisopropylatrazine, desethylatrazine and diaminochlorotriazine. Urinary atrazine and metabolite concentrations were adjusted for creatinine concentration. Undetectable concentrations for atrazine and metabolites were set to the LOD divided by $\sqrt{2}$ (Hornung and Reed, 1990). Analyses for residential tap water analytes and urinary atrazine and atrazine metabolites were conducted by averaging their paired measurements and dichotomizing them at their LOD divided by $\sqrt{2}$. The estimated 'doses' for atrazine and chlorotriazines were defined as the product of the volume of unfiltered water ingested per day times the concentration of each analyte in the municipal and tap drinking water.

Levels of atrazine, desisopropylatrazine, desethylatrazine and diaminochlorotriazine were monitored at the Gillespie and Mount Olive municipal water plants by Syngenta Crop Protection, Inc. (Wilmington, DE) as mandated (U.S. Environmental Protection Agency, 2006). Municipal water in these Illinois communities was monitored weekly from April through July and once every two weeks from August through March of 2003–2007. Prior to June 2005, municipal water samples were analyzed by Syngenta using enzyme immunoassay (EIA), and samples containing more than 3.0 μg/L analyte were typically reanalyzed using gas chromatographic/mass selective detection (GC/MS). After June 2005, municipal water samples were analyzed using HPLC-MS/MS (B. Christensen, Syngenta Crop Protection, Inc., personal communication to Illinois Environmental Protection Agency (EPA), February, 2009). Results were reported as concentrations of atrazine and total chlorotriazines (sum of atrazine, desisopropylatrazine, desethylatrazine and diaminochlorotriazine). The limits of quantitation ranged from 0.05 to 0.50 μg/L (Merritt, 2006). The municipal water suppliers of Waterbury and Fair Haven, Vermont, have waivers from the state for monitoring synthetic organic chemicals (SOCs), including atrazine. Waivers are issued when SOCs have never been detected in a water supply and continue as long as no changes in land use in the source protection area occur (J Siriano, personal communication, March 2006). Municipal monitoring data for atrazine and chlorotriazines from the Mount Olive and Gillespie, Illinois, municipal water plants were also used as individual exposure variables. Monitoring data were typically unavailable for each woman's exact date of participation. Therefore, the two municipal plant results that bracketed the participation date were averaged and weighted according to the number of days from that date. The final imputed values were then multiplied by the volume of unfiltered tap water ingested per day to calculate their estimated 'dose' from this source and dichotomized using a median split for analysis.

2.3. Outcome assessment

Questionnaire data were used to assess menstrual cycle length irregularity by asking "Generally speaking, are your periods regular or irregular? That is, is the length of time between the first day of one period and the first day of the next about the same each cycle?". Severe length irregularity was assessed by asking participants if "During the last 12 months, did you ever go for more than 6 weeks without having a menstrual period? Please do not count times when you were pregnant, breastfeeding or using birth control pills?". Menstrual cycle length was categorized by questionnaire respondent as ≤ 24 days, 25–30 days, 31–35 days, 36–42 days and ≥ 43 days (Farr et al., 2004). For statistical analysis, menstrual cycle length was further dichotomized as ≤ 30 days and > 30 days. Intermenstrual bleeding and dysmenorrhea were also assessed.

Menstrual cycle length was also assessed using data from the prospective menstrual cycle diaries in which women ($n=67$) recorded the absence or presence and relative amount of bleeding through two complete menstrual bleeding periods.

A subset of participants ($n=35$) collected daily first morning urine voids for reproductive endocrine assessments beginning the day after their entry interview and continued through the third day after the end of their second study bleeding period. Urine samples were stored in polypropylene vials with glycerol (7% final dilution) to prevent loss of gonadotropin activity when frozen (Kesner et al., 1995). Participants stored urine samples in their home freezer. At the end of collection, they shipped the frozen urine samples on ice packs in Styrofoam to the National Institute for Occupational Safety and Health (NIOSH) Reproductive Endocrinology Laboratory by next-day courier at the end of collection. Samples were stored at NIOSH at −80 °C until analyzed (Kesner et al., 1995).

All study cycle samples were assayed for estrone 3-glucuronide ($E_1$3G) and pregnanediol 3-glucuronide (Pd3G), the primary urinary metabolites of estradiol and progesterone, respectively. Based on these measurements, a 7-day window was identified to analyze for LH to define the preovulatory surge. In some cases, additional samples were assayed for LH to better define the surge. All endocrine measurements were adjusted for urinary creatinine concentration. $E_1$3G and Pd3G were assayed using competitive, double-antibody time-resolved fluoroimmunoassays (Kesner et al., 1994b). LH levels were analyzed using a commercial noncompetitive, two-site, time-resolved fluoroimmunoassay modified and validated for urine analysis (Kesner et al., 1994a). Urinary creatinine was measured using a Vitros 250 Chemistry Analyzer (Ortho-Clinical Diagnostics). The intra- and inter-assay coefficients of variation were 8.41% and 10.2% for $E_1$3G, 16.15% and 12.05% for Pd3G, 4.70% and 2.61% for LH, and 1.65% and 0.94% for creatinine, respectively.

Follicular phase length and days of the reproductive endocrine measurements were determined using the first day of menses and day of ovulation. Onset of menses was determined from the diary bleeding records, guided by an algorithm (Hornsby, 1991). In brief, the menstrual cycle and the menstrual period began on the first of 2 consecutive days of bleeding; only one of these 2 days could be spotting. Menses was preceded and followed by ≥ 3 consecutive days of non-bleeding or spotting. After day 2 of the period, 1–2 day intervals of non-bleeding or spotting was counted as part of the menses. Day of ovulation was defined as the day of luteal transition (Baird et al., 1995) or, if that was indeterminate, the day of LH surge onset (Kesner et al., 1998). Due to the short ($\sim$7 day) periovulatory sampling window for LH, the algorithm for day of LH surge onset (Reutman et al., 2002) was modified to allow for up to 5 samples to be missing for the 7-day baseline prior to the LH surge onset. Follicular phase length and four endocrine endpoints were selected as outcomes *a priori* based on evidence they are associated with reduced fertility in ovulatory cycles (Baird et al., 1999). The endocrine endpoints were defined as previously described (Baird et al., 1999; Reutman et al., 2002): preovulatory LH, follicular phase Pd3G, mid-luteal phase Pd3G, and mid-luteal phase $E_1$3G. Follicular phase length was defined as the first day of menses to the day of ovulation. The LH surge peak level was also selected as an *a priori* endpoint due to evidence that atrazine abates the LH surge in rats and pigs (Cooper et al., 2000, 2007; Gojmerac et al., 2004; McMullin et al., 2004; Narotsky et al., 2001; Trentacoste et al., 2001). In contrast to the single LH surge peak value, the preovulatory LH endpoint is the 3-day mean leading up to and, typically including, the first significant elevation of the surge, but not necessarily its peak (Reutman et al., 2002).

2.4. Statistical analysis

Descriptive data were summarized for the total sample and stratified by state of residence. Differences between Illinois and Vermont women were analyzed by *t*-tests for continuous variables on transformed data as appropriate and chi-square tests for categorical variables. Bivariate and multivariate unconditional logistic regression were used to calculate odds ratios (OR) and 95% confidence intervals (CI). The associations between markers of atrazine exposure obtained from questionnaire data (state, years in current home, and unfiltered water consumption) and the five menstrual cycle characteristic outcomes (irregularity, severe irregularity, long/not long, inter-menstrual bleeding and dysmenorrhea) were assessed using Vermont women as the referent group. Potential confounders included age, race, parity, education, income, caffeine, vegetable and fruit consumption, alcohol consumption, current smoking, age at menarche, amount of physical activity, and body mass index. Physical activity was assessed by asking the following question: "During a typical week, how many hours do you spend doing strenuous exercise (heart beats rapidly)? Potential occupational exposure to pesticides was queried. Caffeine consumption was determined by multiplying the number of milligrams of caffeine (coffee=107 mg, tea=34 mg, cocoa=10 mg and soda=47 mg) by the number of cups, glasses or cans of each beverage consumed. The cutpoints for confounders were based on previous findings in the literature or approximate median splits and are presented in Table 1. Variables that changed the OR by 10% or more when added to the logistic model were retained in the final models (Mickey and Greenland, 1989). In addition, age and smoking were retained in all logistic models.

Mean differences in menstrual cycle were analyzed by *t*-tests on transformed data. The relationship between exposure to atrazine and menstrual cycle length (in days) was assessed using bivariate and multivariable linear regression (data not shown).

The relationships between atrazine exposure and the results of analyses of reproductive hormone levels and follicular phase length were analyzed by multivariable linear regression. The assumptions required for linear regression were tested prior to analysis. All hormone data were log transformed; follicular phase length data were subjected to an inverse transformation. Menstrual cycle length data also violated the linear regression assumptions; therefore, it was necessary to do an inverse square transformation on menstrual cycle length data prior to analysis when analyzed continuously. Confounding was evaluated and defined the ability of the factor to induce a clinically meaningful event. Baird et al. (1999) suggested that conception is significantly associated with a one unit increase in each of the endocrine endpoints. Therefore, potential confounders that changed the β coefficient by more than one unit when added to the linear regression model were retained in the final model. Potential confounders which changed the statistical significance (at $p=0.05$) of follicular phase length were also retained for the final model. The variables age and smoking were retained in all linear models. Statistical analyses were performed using Statistical Analysis Software (SAS) (Version 9.1.3, SAS Institute, Inc., Cary, NC).

3. Results

A total of 1826 recruitment phone calls (976 in Illinois and 850 in Vermont) were made. Of the recruitment call attempts, 184 resulted in nonworking phone numbers and 402 failed to reach an individual. No eligible participants resided at 1022 of the remaining 1240 households called. The overall participation rates (including eligible and ineligible women contacted) did not differ by state (Illinois=5.43%, Vermont=5.76%; $p=0.76$). Of the 102 women who completed the questionnaire (Illinois=53, Vermont=49), 67 prospectively maintained a menstrual cycle diary (Illinois=30, Vermont=37). Among this group, 38 women also provided daily urine samples. Of these, three women were lost to follow-up, resulting in a final sample of 35 participants for hormone analyses (Illinois=15, Vermont=20). The participation rates for women who completed daily diaries and provided daily urine samples were similar between states.

Analyses of demographic variables are presented in Table 1 for the study sample and stratified by state. Vermont women were older than Illinois women (35.1 years vs. 32.6 years, $p=0.01$) and more educated (49.0% college graduates vs. 30.2%, $p=0.05$). There were no statistically significant differences between Vermont and Illinois women with regards to BMI, income, smoking status, consumption of alcohol, caffeine, vegetables, fruit or physical activity. None of the women reported currently working with pesticides at her job (data not shown).

Questionnaire data for exposure and menstrual cycle characteristics are presented in Table 2. There were no statistically significant differences between Vermont and Illinois women for the number of years they lived in their current homes, amount of unfiltered water consumed, dysmenorrhea, menstrual cycle length and inter-menstrual bleeding. Menstrual cycle length irregularity was reported more frequently in Illinois than in Vermont ($p=0.003$). Illinois women were also more likely to report going more than six weeks without a menstrual period ($p=0.01$).

3.1. Exposure assessment

For both states combined, 35 tap water samples contained detectable levels of atrazine (43%) and chlorotriazines (29%). In Illinois, atrazine and chlorotriazines levels exceeded the LOD in 15 and 6 of the 15 tap water samples, respectively. None of the 20 Vermont tap water samples exceeded the atrazine LOD; four samples exceeded the chlorotriazines LOD. None of the 35 tap water samples contained atrazine at a concentration above the U.S. EPA maximum contaminant level (MCL) of 3.0 μg/L; the maximum atrazine level was 0.95 μg/L. Tap water atrazine levels were twice as high in Illinois than in Vermont (0.7 vs. 0.4 μg/L, $p \leq 0.001$), while chlorotriazine levels were lower in Illinois than in Vermont (2.5 vs. 3.3 μg/L).

Data from the atrazine monitoring program in Illinois were available for 2003–2005 and 2007; data for 2006 were not available (Syngenta Crop Protection, Inc). Of these years, average annual finished drinking water levels for atrazine and chlorotriazines were lowest in 2005. The average atrazine concentrations during the study period (July 13, 2005–September 18, 2005) were 0.16 μg/L in Mount Olive and 0.36 μg/L in Gillespie (data not

Table 1
Demographic characteristics of the study population in Vermont and Illinois.

	Vermont		*Illinois*	
Characteristics	***N=49***	***Percent***	***N=53***	***Percent***
Age (years), Mean ± SD	35.1 (3.9)		32.6 (5.6)**	
Body mass index				
< 25	25	52.1	22	41.5
≥ 25	23	47.9	31	58.5
Education				
Not college graduate	25	51.0	37	69.8*
College graduate	24	49.0	16	30.2
Income				
< $60,000	31	64.6	33	70.2
≥ $60,000	17	35.4	14	29.8
Current smoker				
No	40	81.6	37	69.8
Yes	9	18.4	16	30.2
Alcohol consumption (times per week)				
< 1	23	46.9	33	62.3
≥ 1	26	53.1	20	37.7
Caffeine consumption (mg per day)				
< 300	40	75.5	41	83.7
≥ 300	13	24.5	8	16.3
Vegetables (servings per day)				
≤ 1	17	34.7	21	39.6
> 1	32	65.3	32	60.4
Fruit (servings per day)				
≤ 1	30	61.2	33	62.3
> 1	19	38.8	20	37.7
Weekly physical activity (h)				
0–3	23	46.9	28	52.8
> 3	26	53.1	25	47.2

SD, standard deviation.

* $p \le 0.05$.

** $p \le 0.01$; p-value comparing characteristics between Vermont and Illinois women.

shown). For the chlorotriazines, the average concentrations were 0.53 μg/L in Mount Olive and 0.96 μg/L in Gillespie. Relative to 2005, municipal plant annual average atrazine levels in Mount Olive were 20 times higher in 2003, seven times higher in 2004, and nine times higher in 2007. Annual average atrazine levels were approximately twice as high in Gillespie in 2003, 2004 and 2007 as in 2005 (data not shown).

Desethylatrazine mercapturate was detectable in 67% of the urine samples. Atrazine and all other metabolites were detectable in only 0–31% of urine samples, depending on the analyte. Therefore, only desethylatrazine mercapturate concentrations were examined as a urinary exposure variable, and was not different between states (Table 1). "Doses" of atrazine and chlorotriazine, based on tap water measurements, were also not different between states. "Dose" based on municipal water atrazine levels were only calculable for Illinois (Table 1).

3.2. Menstrual cycle characteristics

Relationships between two measures of menstrual cycle length irregularity and three markers of atrazine exposure are presented in Table 3. Menstrual cycle length irregularity was significantly associated with all three exposure indices when compared to Vermont: residence in Illinois (OR=4.69; 95% CI: 1.58–13.95), residing >4 years in current Illinois home (OR=6.88; 95% CI: 2.08–22.78), and amount of unfiltered water consumed (≤2 cups: OR=4.10, 95% CI: 1.24–13.51; >2 cups: OR=5.73, 95% CI: 1.58–20.77). These associations remained significant after adjusting for confounders (Table 3).

Menstrual cycles longer than 6 weeks were also significantly more prevalent in women whose state of residence was Illinois vs. Vermont (OR=6.16; 95% CI: 1.29–29.38). This measure of cycle irregularity also tended to occur more frequently in women living in Illinois or consuming unfiltered Illinois water, compared to Vermont women. However, results were based on small numbers and there was no evidence of a dose response with years in the home or cups of unfiltered water consumed daily (Table 3). Multivariable analyses were not conducted due to the small sample size for this outcome.

None of these three indices of atrazine exposure derived from the questionnaire were significantly associated with inter-menstrual bleeding, menstrual cycle length (long/not long) or dysmenorrhea. There were also no statistically significant differences between any of the atrazine exposure variables and mean menstrual cycle lengths derived from the prospective diary. There were also no statistically significant associations between atrazine and menstrual cycle length based on linear regression analyses.

3.3. Reproductive hormone analyses

There were no statistically significant differences for any of the urinary reproductive hormones by state of residence, years in the current home or volume of unfiltered Illinois water consumed (data not shown), although levels for all hormones were consistently lower in Illinois women. Mean mid-luteal phase $E_1$3G levels were inversely related to several atrazine exposure indices (low vs. high): residential tap water levels of atrazine (31.3 ng/mg creatinine (Cr) vs. 24.2 ng/mg Cr, $p=0.11$), atrazine 'dose' and chlorotriazines 'dose,' calculated from tap water levels (35.7 ng/mg Cr vs. 24.4 ng/mg Cr, $p=0.01$, for both analytes), and chlorotriazines 'dose' calculated from municipal water levels (33.0 ng/mg Cr vs. 20.9 ng/mg Cr, $p=0.09$) (data not shown).

Mean mid-luteal phase Pd3G levels were inversely related to all four estimated 'doses' of atrazine and chlorotriazines in tap and municipal water, but were significant only for atrazine 'dose' in municipal water (12.4 μg/mg Cr vs. 7.9 μg/mg Cr, $p=0.02$). Paradoxically, Pd3G levels were directly related to chlorotriazine levels in tap water (9.5 μg/mg Cr vs. 12.7 μg/mg Cr, $p=0.11$) (data not shown).

Relationships between exposure indices and preovulatory and surge peak levels of LH were inconsistent. The mean LH surge peak decreased with increases in each of the estimated 'dose' variables, but only those based on municipal chlorotriazine levels approached significance (63.3 mIU/mg Cr vs. 43.6 mIU/mg Cr, $p=0.11$). Conversely, LH surge peak levels were directly related to municipal atrazine levels (44.5 mIU/mg Cr vs. 77.7 mIU/mg Cr; $p=0.03$) and residential chlorotriazines levels (46.4 mIU/mg Cr vs. 66.9 mIU/mg Cr; $p=0.005$). There were no significant relationships between mean preovulatory LH levels and the atrazine exposure variables (data not shown).

β coefficients for the relationship between markers of atrazine exposure and log-transformed hormone concentrations are presented in Table 4. Urinary mid-luteal phase $E_1$3G levels tended to be lower in Illinois residents ($\beta=-0.32$; 95% CI: −0.68–0.04), women who had lived in their current Illinois home more than four years ($\beta=-0.43$; 95% CI: −0.87–0.02), and women with higher tap water atrazine levels ($\beta=-0.32$; 95% CI: −0.68–0.04). Mid-luteal phase $E_1$3G was strongly associated with the 'dose' of atrazine and chlorotriazines in tap water ($\beta=-0.46$; 95% CI: −0.82–−0.10 for both analytes).

Table 2
Exposure and menstrual cycle characteristics of the study population in Vermont & Illinois.

	Vermont		***Illinois***	
	N=49	***Percent***	***N=53***	***Percent***
Years in current home				
<4	18	36.7	24	45.3
≥ 4	31	63.3	29	54.7
Cups per day of unfiltered water				
≤ 2	32	65.3	32	60.4
>2	17	34.7	21	39.6
Residential tap water, Mean $\pm$ SD				
Atrazine (ppb)	0.4 (0.0)		0.7 (0.2)**	
Chlorotriazine (ppb)	3.3 (3.2)		2.5 (0.7)	
Urinary biomarker, Mean $\pm$ SD				
(Desethylatrazine mercaptureate)	11.3 (40.0)		9.0 (28.5)	
Dose[a], Mean $\pm$ SD				
Atrazine (municipal)			0.4 (0.3)	
Chlorotriazine (municipal)			1.3 (1.0)	
Atrazine (residential)	0.9 (0.9)		2.0 (1.5)	
Chlorotriazine (residential)	7.6 (8.6)		6.8 (5.1)	
Dysmenorrhea				
Never or sometimes	16	32.7	23	43.4
Often or always	33	67.4	30	56.6
Menstrual cycle length regularity				
Yes	43	89.6	33	64.7**
No	5	10.4	18	35.3
Cycle length (days)				
<24	4	8.7	2	3.8
25–30	36	73.5	38	71.7
31–35	6	12.2	6	11.3
36–42	1	2.0	3	5.7
>43	0	0.0	1	1.9
Too irregular to say	2	4.1	3	5.7
Did you ever go more than 6 weeks without a menstrual period?				
Yes	2	4.1	11	20.8
No	47	95.9	42	79.3
Did you ever bleed or spot between menstrual periods?				
Yes	5	10.2	8	15.1
No	44	89.8	45	84.9
Follicular phase length (days), Geometric mean $\pm$ SD	15.3 (1.3)		15.3 (1.3)	
Urinary reproductive hormones, Geometric mean $\pm$ SD				
Preovulatory LH (mIU/mg Cr)	16.2 (1.7)		12.56 (2.0)	
Mid-luteal phase $E_1$3G (ng/mg Cr)	28.7 (1.6)		20.83 (1.8)	
Follicular phase Pd3G (μg/mg Cr)	0.8 (1.9)		0.8 (1.6)	
Mid-luteal phase Pd3G (μg/mg Cr)	9.3 (1.8)		8.8 (1.7)	
LH surge (mIU/mg Cr)	51.9 (1.4)		44.7 (1.6)	

SD, standard deviation.

[a] Doses were calculated by multiplying the volume of unfiltered water ingested per day by the concentration of atrazine or chlorotriazine in municipal water or residential tap water.

** $p \leq 0.01$; p-value comparing characteristics between Vermont and Illinois women.

Mid-luteal phase Pd3G levels were inversely and significantly associated with atrazine 'dose' when calculated using municipal plant data ($\beta = -0.57$; 95% CI: -1.06–-0.09). Although not statistically significant, inverse associations were also present with estimated 'dose' of municipal water chlorotriazines ($\beta = -0.43$; 95% CI: -1.03–0.18) and tap water atrazine and chlorotriazines ($\beta = -0.31$; 95% CI: -0.71–0.08 for both analytes).

The results for LH levels remained inconsistent in regression analyses (Table 4). LH surge peak levels remained directly associated with chlorotriazine levels in tap water ($\beta = 0.39$; 95% CI: 0.10–0.68) and with atrazine levels in municipal water ($\beta = 0.64$; 95% CI: -0.08–1.37). None of the atrazine exposure markers was significantly associated with preovulatory LH levels. There was little evidence of an association between follicular phase Pd3G levels and any of the atrazine exposure variables (data not shown).

3.4. Follicular phase length analyses

No statistically significant relationships were found between any of the markers of atrazine exposure and mean follicular phase length. For linear regression analyses, follicular phase length data were inversely transformed and parity was included as a covariate in all models. Follicular phase length was significantly longer with elevated chlorotriazines levels in municipal water ($\beta = -0.019$; 95% CI: -0.04–0.00) and with elevated 'dose' of atrazine in municipal water (adjusted: $\beta = -0.018$; 95% CI: -0.04–0.00) (data not shown).

Table 3
Relationship between markers of atrazine exposure and menstrual cycle characteristics as reported by retrospective questionnaire.

Outcome	Exposure	Crude				Adjusted			
		Cases, controls	OR	95% CI	*P*-value	Cases, controls	OR	95% CI	*P*-value
Menstrual Cycle Length Irregularity	State of residence								
	Vermont	18.33	1.00	–	–	4.43	1.0	-	-
	Illinois	5.43	4.69	1.58, 13.95	0.005	18.33	4.32[a]	1.27, 14.63	0.02
	Years in current home								
	Vermont	4.43	1.00	–	–	4.42	1.0	–	–
	≤4 (Illinois)	6.18	2.87	0.78, 10.60	0.11	5.17	1.94[b]	0.41, 9.24	0.41
	>4 (Illinois)	12.15	6.88	2.08, 22.78 p for trend: 0.002	0.002	11.13	8.55[b]	2.15, 33.91 p for trend: 0.003	0.002
	Cups per day of unfiltered water								
	Vermont	5.43	1.00	–	–	4.43	1.00	–	–
	≤2 (Illinois)	10.21	4.10	1.24, 13.51	0.02	10.21	5.41[c]	1.34, 21.84	0.02
	>2 (Illinois)	8.12	5.73	1.58, 20.77 *p* for trend: 0.005	0.01	8.12	6.73[c]	1.37, 33.07 p for trend: 0.01	0.02
>6 weeks between menstrual bleedings	State of residence								
	Vermont	2.47	1.00	–	–		**	–	–
	Illinois	11.42	6.16	1.29, 29.38	0.02				
	Years in current home								
	Vermont	2.47	1.00	–	–		[d]	–	–
	≤4 (Illinois)	7.17	9.68	1.83, 51.22	0.01				
	>4 (Illinois)	4.25	3.76	0.64, 21.97 p for trend: 0.12	0.14				
	Cups per day of unfiltered water								
	Vermont	2.47	1.00	–	–		[d]	–	–
	≤2 (Illinois)	8.24	7.83	1.54, 39.81	0.01				
	>2 (Illinois)	3.18	3.92	0.60, 25.41 p for trend: 0.09	0.15				

OR, odds ratio; CI, confidence interval.

[a] Adjusted for age (continuous), BMI (<25/≥25) and current smoker (yes/no).
[b] Adjusted for age (continuous), BMI (<25/≥25), income (<$60,000/≥$60,000) and current smoker (yes/no).
[c] Adjusted for age (continuous), BMI (<25/≥25), education (college graduate/non college graduate) and current smoker (yes/no).
[d] Frequencies <4, adjusted ORs not calculated.

4. Discussion

To our knowledge, this study is the first to examine potential associations between exposure to atrazine in drinking water and menstrual cycle characteristics and reproductive hormone levels, and the first to report effects of this pesticide on human hormone concentrations. Despite unusually low atrazine levels in the Illinois drinking water during the summer of 2005, significant associations with disrupted menstrual cycle function were detected. Potentially important findings were that several different indicators of atrazine exposure were significantly associated with reduced mid-luteal phase levels of $E_1$3G, the major urinary metabolite of estradiol that is well correlated with circulating estradiol, the primary estrogen in humans (O'Connor et al., 2003). Our results are consistent with some (Coady et al., 2005; Gojmerac et al., 1996; Gojmerac et al., 1999; Mitak et al., 2001), but not all (Cummings et al., 2000; Wetzel et al., 1994) of the literature for females from a variety of species.

Most studies have shown atrazine does not have intrinsic estrogenic activity and does not bind to the estrogen receptor (Cooper et al., 2007; Eldridge et al., 1999; Tennant et al., 1994). Yet, some toxicological findings suggest atrazine may be anti-estrogenic and inhibit estrogen-stimulated responses (Cooper et al., 2007; Tennant et al., 1994). Our results suggest that atrazine may be functionally anti-estrogenic in women.

Health effects associated with reduced circulating estrogen have been widely reported and include compromised implantation, reduced fertility (Baird et al., 1999) and impaired general health including osteoporosis (Turner et al., 1994), cardiovascular disease (Mendelsohn and Karas, 1999) and central nervous system deterioration (Nappi et al., 1999; Sherwin, 2006).

We provide evidence that atrazine exposure is also associated with reduced mid-luteal phase Pd3G levels. Reduced mid-luteal levels of both $E_1$3G and Pd3G are associated with impaired conception (Baird et al., 1999). Reduced Pd3G levels during the luteal phase reflect reduced progesterone secretion by the corpus luteum and reduced luteal function. Insufficient progesterone and luteal phase deficiency (Soules et al., 1989) impair implantation and lead to infertility and repeated spontaneous abortions (McNeely and Soules, 1988).

We found no compelling evidence that atrazine was associated with altered LH secretion before and during the preovulatory surge as has been shown in rats and pigs. This difference in our findings compared to those in animals may be due to the relatively low levels of atrazine exposure in our study and/or insufficient statistical power. Alternatively, this may reflect a species difference in the action of atrazine, since the mechanism whereby estrogen triggers the preovulatory LH surge differs among these species (Kesner, 1988).

Another finding of this study was the association between menstrual cycle length irregularity and several indices of atrazine exposure. Two different (albeit not independent) measures of increased cycle length irregularity were associated with living in Illinois, living for more than 4 years in Illinois, and drinking increasing amounts of unfiltered water in Illinois.

We detected no significant associations between indices of atrazine exposure and menstrual cycle length as assessed by both

Table 4
Linear regression analyses of markers of atrazine exposure and reproductive hormone concentrations.

Exposure	Mid-luteal estrone 3-glucuronide[a]					Mid-luteal pregnanediol 3-glucuronide[a]					Luteinizing hormone surge peak[a]					Preovulatory luteinizing hormone[a]				
	n	*β*	95% CI		*P*-value	*n*	*β*	95% CI		*P*-value	*n*	*β*	95% CI		*P*-value	*n*	*β*	95% CI		*P*-value
State of residence																				
Vermont	20					20					19					20				
Illinois	15	−0.33	−0.72,	0.06	0.09	15	1.00	−0.41,	0.42	1.00	14	−0.18	−0.49,	0.12	0.23	13	−0.31	−0.75,	0.14	0.17
Years in current home (Illinois & Vermont)																				
Vermont	20					20					19					20				
≤ 4 (Illinois)	7	−0.22	−0.72,	0.28	0.37	7	0.14	−0.39,	0.67	0.59	6	−0.24	−0.65,	0.16	0.23	6	−0.21	−0.78,	0.37	0.47
> 4	8	−0.42	−0.90,	0.06	0.08	8	−0.12	−0.63,	0.38	0.62	8	−0.14	−0.51,	0.24	0.46	7	−0.40	−0.96,	0.17	0.15
Cups per day of unfiltered water (CI, confidence interval)[b]																				
Vermont	20					20					19					20				
≤ 2	6	−0.40	−0.93,	0.13	0.13	6	0.24	−0.31,	0.79	0.39	6	−0.26	−0.67,	0.16	0.22	5	−0.18	−0.81,	0.46	0.57
> 2	9	−0.28	−0.74,	0.18	0.23	9	−0.16	−0.64,	0.32	0.50	8	−0.13	−0.50,	0.23	0.46	8	−0.39	−0.91,	0.13	0.14
Residential tap water[c]																				
Atrazine																				
≤ 0.36	20					20					19					19				
> 0.36	15	−0.33	−0.72,	0.06	0.09	15	0.00	−0.41,	0.42	0.99	14	−0.18	−0.49,	0.12	0.23	14	−0.31	−0.75,	0.14	0.17
Chlorotriazine																				
≤ 2.50	25					25					24					23				
> 2.50	10	0.21	−0.22,	0.63	0.32	10	0.34	−0.08,	0.76	0.11	9	−0.41	0.11,	0.71	0.01	10	0.30	−0.18,	0.79	0.21
Urinary biomarker (desethylatrazine mercapturate)																				
≤ 0.36	12					12					12					12				
> 0.36	23	0.04	−0.39,	0.46	0.86	23	−0.03	−0.46,	0.40	0.90	21	0.07	−0.25,	0.40	0.65	21	0.08	−0.40,	0.55	0.75
Municipal water supply[d]																				
Atrazine																				
≤ 0.20	13					12					11					12				
> 0.20	2	0.33	−0.74,	1.39	0.51	1	−0.20	−1.06,	0.66	0.63	2	0.62	−0.18,	1.42	0.11	2	0.17	−1.03,	1.38	0.75
Chlorotriazine																				
≤ 0.43	10					10					8					9				
> 0.43	5	0.15	−0.69,	0.99	0.70	5	0.08	−0.59,	0.76	0.79	5	0.08	−0.63,	0.80	0.80	5	−0.30	−1.00,	0.93	0.94
Dose[e]																				
Atrazine (municipal)																				
≤ 0.20	5					5					4					5				
> 0.20	9	−0.54	−1.25,	0.16	0.12	9	−0.70	−1.12,	−0.27	0.004	8	−0.04	−0.70,	0.62	0.89	8	−0.17	−1.80,	0.74	0.68
Chlorotriazine (municipal)																				
≤ 0.43	3					3					2					3				
> 0.43	11	−0.61	−1.35,	0.14	0.10	11	−0.51	−1.09,	0.08	0.08	10	−0.39	−1.05,	0.28	0.22	10	−0.42	−1.41,	0.58	0.37
Atrazine (residential tap water)																				
≤ 0.36	11					11					10					10				
> 0.36	23	−0.49	−0.86,	−0.12	0.01	23	−0.37	−0.77,	0.02	0.06	22	−0.10	−0.44,	0.24	0.54	22	−0.18	−0.66,	0.29	0.43
Chlorotriazine (residential tap water)																				
≤ 2.50	11					11					10					10				
> 2.50	23	−0.49	−0.86,	−0.12	0.01	23	−0.37	−0.77,	0.02	0.06	22	−0.10	−0.44,	0.24	0.54	22	−0.18	−0.66,	0.29	0.43

CI, confidence interval.

[a] Log transformed and adjusted for age (continuous) and current smoker (yes/no).
[b] Assessed by the question "During a typical day while at home, how many (unfiltered) glasses of plain water (and powdered or concentrated water) do you drink at home?".
[c] Average of the two residential tap water samples dichotomized at limit of detection/$\sqrt{2}$.
[d] A temporally weighted average of the two municipal monitoring results closest to the date of participation for each woman. Dichotomized using a median split.
[e] Doses were calculated by multiplying the volume of unfiltered water ingested per day by the concentration of atrazine or chlorotriazine in municipal water or residential tap water.

retrospective questionnaire and prospective menstrual bleeding diaries. However, the present study does suggest that atrazine exposure may increase follicular phase length, supporting previous research (Farr et al., 2004). We found that 'dose' of atrazine and levels of chlorotriazines, both based on municipal water measurements, were significantly associated with increased follicular phase length. Cycles with a longer follicular phase are associated with reduced probability of conception (Baird et al., 1999). Our findings of increased follicular phase length are consistent with toxicological studies, which show that atrazine exposure alters estrous cyclicity in rats (Agency for Toxic Substances and Disease Registry, 2003; Cooper et al., 2007; Laws et al., 2000; Simic et al., 1994; Wetzel et al., 1994).

Previous epidemiologic studies have reported associations between endocrine disruptors and increased cycle length. Exposure to organic solvents was associated with increased cycle length (>35 days) in a cross-sectional study of 1408 petrochemical workers in China (Cho et al., 2001). Exposures to PCBs (Cooper et al., 2005) and dioxin (Eskenazi et al., 2002) were each associated with increased menstrual cycle length.

It is not clear why atrazine exposure would disrupt menstrual cycle length regularity, follicular phase length and ovarian hormone secretion without altering overall cycle length. One possibility is that the initial disruption of cycle length is not to consistently shorten or lengthen the cycle, but to disrupt its length in either direction, thereby increasing cycle irregularity without changing the central tendency. This is often the case as women enter and then depart their reproductive years at puberty and menopause (Kaufert, 1980; Treloar et al., 1967). Atrazine concentrations may also have been too low to cause a consistent change in menstrual cycle length since prospective diary data reflected atrazine levels during the study when they were low as compared to previous years used in assessments with questionnaire data. Finally, we do not know the latency between exposure and effect. The differential effects may also be a function of the interaction between atrazine dose and its latency and duration of action.

Data obtained from municipal monitoring by both Syngenta Crop Protection, Inc. and the Illinois EPA confirmed that atrazine concentrations in the drinking water of the two Illinois study communities were much lower in 2005 than they were in previous and subsequent years, most likely due to drought conditions (National Drought Mitigation Center, 2005). Only about 43% of our tap water samples had atrazine levels above the LOD (0.5 μg/L) and only 5% of the participants (2 out of 38 women) had measurable urinary atrazine levels. This suggests that most women had not been recently exposed since the half-life of atrazine in water is approximately 6 months (Agency for Toxic Substances and Disease Registry, 2003).

The predominant metabolite detected in urine in this study was desethylatrazine mercapturate. The presence of this metabolite could indicate exposure to the parent compound or to one of the dealkylated atrazine breakdown products which, along with atrazine, are biologically active (Barr et al., 2007). However, the short half-life (approximately eight hours) and limited bioaccumulation of atrazine (Catenacci et al., 1993) and its metabolites in humans limit the usefulness of biomonitoring, since levels represent only recent exposures (Barr et al., 2006). Accordingly, we assessed atrazine exposure by a variety of direct and indirect indices.

The overall participation rate was low, due primarily to eligibility requirements (women 18 and 40 years old not taking oral contraceptives) and the requirements to collect multiple biological samples and maintain a diary. These factors resulted in a small sample size, which in turn, reduced statistical power and precision. Participation rates and the self-reported rates of infertility, number of pregnancies, number of live births and menstrual cycle characteristics (dysmenorrhea, inter-menstrual bleeding and menstrual cycle length) were similar for women between the two states, suggesting that selection bias was unlikely. Women were not informed about the specific hypotheses of the study, making it unlikely that differential recall biased the results.

It is possible that some findings were the result of factors related to state of residence or to unmeasured drinking water contaminants such as another water soluble pesticide; e.g. 2,4-dichlorophenoxyacetic acid which is also used on corn. It is also possible that a plasticizing agent (e.g., a phthalate) leached from a storage container or a disinfection byproduct was responsible for the findings. In an effort to ensure the water systems had similar background levels of unmeasured contaminants, rural communities using surface water were chosen in both states. Levels of trihalomethanes and haloacetic acids in municipal water systems did not exceed their respective standards. An additional limitation of this study is that generalizability of these results is limited since the participants were predominately white and middle-class women with some college education who lived in rural communities.

In summary, we report evidence that exposure to atrazine in municipal drinking water, at levels below the U.S. EPA MCL for chronic exposure of 3.0 ppb (as well as the shorter 90-day average exposure of 37.5 ppb and the one-day concentration of 298 ppb), is associated with menstrual cycle length irregularity, reduced reproductive hormone levels and longer follicular phase in women. Our results are consistent with the literature and with biological processes of the hypothalamic–pituitary–ovarian axis as it may relate to plausible mechanism(s) of action. Given the interdependence of the reproductive hormone axis, modifications in the levels of one hormone may lead to other endocrine changes, reduce fertility and result in endocrine-related conditions (Scialli and Zinaman, 1993).

Results of this study are preliminary and further studies on larger populations are needed to confirm and extend these findings. Additionally, relationships between atrazine exposure and other reproductive disorders and reproductive hormone-sensitive conditions should be explored.

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