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Effects of gestational and lactational exposure to heptachlor epoxide on age at puberty and reproductive function in men and women ☆, ☆☆, ☆☆☆

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ABSTRACT

Background: For 15 months in 1981–1982, the commercial milk supply on the Hawaiian island of Oahu was contaminated with heptachlor epoxide, a metabolite of the insecticide heptachlor, resulting in gestational and/or lactational exposure to offspring of women who drank cow milk during that period.

Objective: To determine whether gestational and lactational exposure to heptachlor epoxide alters reproductive function and age at puberty in men or women.

Methods: 457 participants were recruited from a prior high school enrollment sampling frame of 20,000 adults born during 1981–1982 who lived on Oahu since at least first grade. Number of glasses of cow milk consumed weekly by the mother during the participant's gestation was used as a surrogate measure of heptachlor epoxide exposure. Reproductive function measures included semen analyses; reproductive hormones or their metabolites in daily urine specimens for one menstrual cycle; serum reproductive hormone levels in both sexes; and reported ages of onset for pubertal milestones.

Results: We observed no strong associations of heptachlor epoxide exposure during gestation and lactation with reproductive endpoints. In females, heptachlor epoxide exposure was associated with longer luteal phase length and slower drop in the ratio of estradiol to progesterone metabolites after ovulation. In males, heptachlor epoxide exposure was weakly associated with higher serum follicle stimulating hormone and luteinizing hormone concentrations, but no dose–response relationship was apparent.

Conclusions: The results provide limited evidence that gestational and lactational exposure to heptachlor epoxide, due to milk contamination on Oahu in 1981–1982, resulted in clinically significant disturbances of reproductive function in men or women.

1. Introduction

Heptachlor is a chlorinated cyclodiene insecticide that was widely used from the 1950s until the 1980s for the control of termites and soil insects. In mammals, heptachlor is metabolized to heptachlor epoxide, which has comparable toxicity, but is more stable in biological systems (ATSDR, 2007). Heptachlor and heptachlor epoxide are lipophilic, which allows them to readily cross the placenta (ATSDR, 2007). Lactation is a major route of excretion in female mammals, and can serve as another route of transfer of heptachlor epoxide from mother to offspring (ATSDR, 2007).

Since 1987, the only permitted commercial use of heptachlor products in the United States (US) has been to control fire ants in power transformers and in underground cable television and telephone cable boxes. Heptachlor has not been manufactured in the US since 2000 (ATSDR, 2007). Serum heptachlor epoxide levels decreased in the US population between 1999–2000 and 2003–2004 (NHANES,

Abbreviations: $E_1$3G, estrone 3-glucuronide; FSH, follicle stimulating hormone; LH, luteinizing hormone; Pd3G, pregnanediol 3-glucuronide

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2009). While heptachlor has been banned, severely restricted or unregistered[3] in 68 countries (PANNA, 2004), it is still manufactured and used internationally (Baekeland, 2009).

In 1980–1982, pineapple tops, which had been sprayed with heptachlor, were fed to cows on Oahu, contaminating the entire milk supply on the Hawaiian island for 15 months until the issue was discovered in March 1982. During this period, humans were exposed to heptachlor epoxide in cow milk at levels about 10-fold greater than the US Food and Drug Administration action level of 100 ng/g lipid (Baker et al., 1991). There were less than two dozen dairy farms and only two dairies on Oahu at the time. All the milk from cows throughout the island was brought to these two dairies and mixed, so contamination of the commercial milk supply was uniform. There was no or very minimal importation of milk to the island because of legal restrictions at that time on importation of milk to protect the local dairy industry. The contamination was known to be of 15 months duration because stored milk samples from a radiation fallout testing program were tested to discover the start of contamination, and the contamination ceased in March 1982 when it was discovered. Lactating women who consumed dairy products from local sources had mean heptachlor epoxide levels in their breast milk of 123 ng/g lipid, with maximum levels above 250 ng/g lipid (Baker et al., 1991). By comparison, concentrations in breast milk of European women averaged 2.19–2.81 ng/g lipid in 1997–2001 (Shen et al., 2007).

Several health studies of individuals gestationally and lactationally exposed to the contaminated milk on Oahu have been conducted. In an ecologic study of Hawaiian births during the period from 1981 to 1983, based on data from the Birth Defects Monitoring Program, there was no increase in malformations recognized at birth on Oahu, compared to births on the other Hawaiian Islands, births on Oahu before the heptachlor contamination, or births in the general US population (Le Marchand et al., 1986). A study based on birth certificate information did not detect a change on Oahu for fertility, or rates of low birth weight, early or late fetal deaths, or neonatal deaths related to the period of milk contamination (Burch, 1983). However, the study did reveal a transient increase in sex ratio (i.e. greater prevalence of males) among fetal deaths, neonatal deaths, and deaths due to congenital anomalies during the first half of 1982. A longitudinal study evaluated 120 infants born on Oahu in 1982, who were potentially exposed to heptachlor epoxide *in utero* and via breast milk (Hoffman, 1985). Heptachlor epoxide levels in breast milk for a subset ($N=69$) of their mothers, which averaged 120 ng/g lipid, were significantly associated with infant low birth weight, gestational age, jaundice, and days in hospital after birth (Hoffman, 1985). Heptachlor epoxide in breast milk was also associated with slower acquisition of behaviors at 4 and 8 months, but not at 18 and 36 months (Hoffman, 1985). Increased mothers' milk consumption during pregnancy tended to be associated with increased learning disabilities and decreased performance on neurobehavioral tests in the high school student offspring potentially exposed to heptachlor epoxide *in utero* and during lactation (Baker and Yang, 2003; Baker et al., 2004).

Effects of early exposure to heptachlor on reproductive system development and function have been investigated in rats. One study evaluated the effects of oral heptachlor dosed to dams from gestational day 12 to postnatal day 7, then directly to the pups (Smialowicz et al., 2001). The other study evaluated the effects of oral doses to dams from gestational day 8 to postnatal day 21 (Lawson and Luderer, 2004). Considering both studies, there were no significant effects of heptachlor treatment on anogenital distance, nipple retention in males, timing of puberty, serum reproductive hormone concentrations, estrous cycling, fertility, ovarian or testicular histology, or sperm counts. Increased early postnatal mortality was observed at the highest dose level in the latter study, and after treatment on gestation days 6–15 in a third study (Narotsky et al., 1995).

Few studies have evaluated the effects of heptachlor exposure on the development of the human reproductive system. We studied individuals accidentally exposed to heptachlor epoxide on Oahu to test the hypothesis that exposure to this insecticide *in utero* and/or by drinking contaminated breast milk as infants alters reproductive system development in young adults. We evaluated the associations between this heptachlor epoxide exposure and sensitive indicators of reproductive function including sperm and semen parameters, urinary reproductive hormones during the menstrual cycle, and serum reproductive hormone concentrations in men and women.

2. Materials and methods

2.1. Participants

The study was approved by the Institutional Review Board of the University of California Irvine. All participants were informed about the study and signed the informed consent form prior to participation in the study.

We recruited participants from among participants in a previous study of neurobehavioral function (Baker and Yang, 2003; Baker et al., 2004) for which a screening survey was administered to 20,448 children in 20 public schools (all but one of the public schools) and 4 private schools on Oahu to identify 3000 eligible students born between July 1981 and June 1982 who had attended school on Oahu since first grade. From these, 445 students were randomly selected for the previous neurobehavioral study; 332 born on Oahu and 113 born elsewhere. In addition, parents of 2655 eligible, non-selected students were contacted by mail-back screening survey or telephone; 1446 parents responded. We attempted to contact these 1891 students and responding parents to recruit the students for the current study; 768 were unreachable. Of the 1123 who were contacted, 354 declined to participate, 367 were not eligible, 51 were lost to follow-up between initial contact and enrollment, and 351 were enrolled. The primary reason that so many individuals could not be contacted was because the participants graduated from high school during the time interval between the earlier neurobehavioral study and this study, and many of them moved away from their parents' home (the contact address) or moved to the mainland USA for college or work. Because the contact information was at least 3–7 years old, we were unable to reach a large number of individuals from the original study database. Therefore, we returned to the original sampling frame of 20,448 people who had completed the eligibility survey for the neurobehavioral study in the fall of 1998. We acquired a database from the Hawaii State Department of Education, which contained the names of 17,568 former students who had attended public schools on Oahu and met our age eligibility requirements; 168 individuals responded to a mass mailing to 9022 of these former students. We enrolled 106 of these; 32 were eligible but were unwilling to participate, and 30 were not eligible. Together we recruited 457 participants for this study: 399 Oahu-born and 58 born elsewhere. The number of participants not born in Oahu was insufficient to serve as a control, and so their data were not included in the primary analyses.

Exclusion criteria included non-English speaker ($<2\%$ of eligible respondents), no longer living on Oahu, autoimmune disorders, liver disease, sarcoidosis, HIV infection or AIDS, multiple sclerosis, tuberculosis, diabetes, thyroid disease, or adrenal disease. Women were excluded if they had breast fed or used an intrauterine device, hormonal contraception, or other hormonal replacement/medication during the past 3 months, or been pregnant in the past 6 months, if they had a history of surgery on their reproductive system (ovaries, tubes, or uterus), pelvic inflammatory disease, or endometriosis. Men were excluded if they had used medications or supplements containing testosterone, estrogen, or anabolic steroids during the past year. Of the total number of individuals who were not eligible, 47.6% no longer lived on Oahu, 38.7% were women using hormonal contraception, 7.8% were women who were pregnant or breastfeeding, 0.4% were women using IUDs, 1.3% were women with endometriosis, pelvic inflammatory disease, or ovarian/uterine surgery, 0.7% had hyperthyroidism, 1.1% had diabetes, and 0.9% had other medical conditions.

The study was conducted from March 2003–2006, when the participants were 20–25 years old. Male participants were compensated $200 and female participants were compensated $300 if they completed all study interviews and biospecimen collection. We provided partial compensation for partial completion.

[3] Unregistered pesticides are pesticides that were withdrawn by the manufacturer, never registered, or whose registration has expired.

2.2. Questionnaire

A study staff member administered a questionnaire to participants addressing ethnicity, current occupation, occupational exposures, medical history, medications, physical activity, and reproductive history. During the same study visit, participants completed a questionnaire addressing ages of recalled pubertal milestones and tobacco, alcohol, and illicit drug use. Men were asked if they had erectile dysfunction or physician-diagnosed history of hypospadias, cryptorchidism, varicocele, and orchitis. Women provided menstrual history and premenstrual symptoms using a Menstrual Distress Questionnaire (Moos, 1968; Moos and Leiderman, 1978). In addition, women maintained a prospective daily diary to document study urine sample collection, vaginal bleeding, sickness, and use of medications or supplements during the menstrual cycle for which they collected daily urine samples.

The biological mother for each participant (or if unavailable, a surrogate with knowledge of the mother's pregnancy with the participant) completed a Parent Questionnaire in-person or via mail-back to provide place(s) of residence during the pregnancy; participant's birthplace, breastfeeding, and diagnosis of cryptorchidism or hypospadias; and mother's past cow milk consumption, pesticide exposure, medications, stress level, and smoking, alcohol, and illicit drug use during pregnancy.

2.3. Puberty endpoints

Participants were asked at what ages they first noticed growth of hair in their axillae, on their legs, and in their pubic area. Women provided the ages they first noticed breast development and had their first menstrual period. Men provided the ages they started shaving, had their first ejaculation or nocturnal emission, and their voice changed. These questions were derived from previously validated puberty self-report instruments (Brooks-Gunn et al., 1987; Gilger et al., 1991).

2.4. Exposure variable

In lieu of biomarkers to assess heptachlor epoxide exposure and a suitable number of controls not born on Oahu, exposure was defined as the number of glasses of cow milk consumed by each Oahu-born participant's mother during their gestation. In a population-based study conducted eight to nine years after the heptachlor epoxide milk contamination, investigators found that self-reported milk consumption was highly associated with serum concentrations of heptachlor epoxide in adults and breast milk concentrations of heptachlor epoxide in newly lactating females controlling for prior breastfeeding, but milk consumption was not associated with other pesticides (Baker et al., 1991). The mothers'-cow-milk-consumption variable was multi-modally distributed, with peaks at 7, 10, 14, and 21 glasses/week, suggesting a digit preference. Therefore, we categorized this variable as 0–5, 6–11, and ≥ 12 glasses/week for the primary analyses. We conducted additional analyses in which milk consumption was modeled as a continuous variable.

2.5. Menstrual cycle function endpoints

Women collected first morning urine samples daily for one menstrual cycle or, in the event of irregular cyclicity or amenorrhea, for ≤ 49 days, beginning on the first day of bleeding, and continuing through the third day of the subsequent period. Participants stored 5 mL aliquots of each sample in their freezer in polypropylene vials containing glycerol to prevent gonadotropin activity loss (Kesner et al., 1995). Samples were retrieved by study coordinators and stored at -18 °C in a University of Hawaii freezer before being shipped to the National Institute for Occupational Safety and Health Endocrinology Laboratory and stored at -80 °C until assayed. Urinary luteinizing hormone (LH), follicle stimulating hormone (FSH), estrone 3-glucuronide ($E_1$3G), and pregnanediol 3-glucuronide (Pd3G) were assayed as previously described (Kesner et al., 1994a, 1994b, 1998). $E_1$3G and Pd3G are the principal metabolites of estradiol and progesterone, respectively, in the urine. Mean intra-assay coefficients of variation for the urinary LH, FSH, $E_1$3G, and Pd3G assays were 6.8%, 7.7%, 8.6%, and 9.9%; mean interassay coefficients of variation were 10.9%, 9.6%, 4.2%, and 5.3%, respectively. These endocrine measurements were divided by urinary creatinine concentration.

We selected fourteen endpoints to assess female reproductive function based on previous studies (Baird et al., 1995; Baird et al., 1991; Baird et al., 1999; Kassam et al., 1996; Reutman et al., 2002): menstrual cycle, follicular phase, and luteal phase lengths; early follicular FSH and $E_1$3G levels, follicular Pd3G level, follicular LH:FSH ratio, pre-ovulatory LH level, ovulation (yes/no), steepest $E_1$3G:Pd3G slope post-ovulation, early–midluteal Pd3G rise, midluteal $E_1$3G and Pd3G levels, and slope of FSH levels before menses. The algorithms used to calculate the variables are summarized in Supplemental Table 1.

We analyzed data from one menstrual cycle per woman. Most women had data from only one cycle. In the rare event of more than one complete cycle, we chose the first one. If both cycles had missing samples, the cycle with the most complete data was chosen. We excluded two cycles with so many missing samples that most variables could not be calculated. We excluded one cycle during which the participant started using oral contraceptives. In some cases, participants appeared to have poured their urine void into consecutive vials yielding unvarying concentrations for all endocrine analytes and creatinine in those consecutive samples. In four of these cases, the entire cycle was excluded; in five other cases a limited number of samples exhibited this analyte pattern and those samples were excluded from the calculation of endpoints.

Occasionally endocrine variables could not be calculated due to missing data on a key day. A panel of the authors (U.L., J.S.K. & J.M.F.) blinded to exposure status decided through detailed discussion and consensus on a case-by-case basis whether to interpolate data or impute variables. Endocrine concentrations for each missing sample was interpolated as the mean of its bracketing samples, but only when (1) the bracketing values were similar to each other and (2) the missing sample occurred during a stage of the cycle when only modest concentration changes are typical, e.g., interpolating Pd3G during the mid-follicular phase. The panel also imputed the following endocrine variables that were apparent but not computed due to missing samples: day of LH surge onset or day of luteal transition ($n=7$); ovulatory status of one truncated cycle; assigning the day of onset of the second menses if the participant stopped collecting urine samples and diary entries ended before the required three days of bleeding.

2.6. Serum reproductive hormones

Blood (10 mL) was collected by venipuncture into a red-top Vacutainer tube. Blood samples were collected between 7 a.m. and noon to minimize variation due to diurnal rhythms. While blood was meant to be collected from women during their luteal phase, 5–9 days before expected menses onset to provide an additional index that ovulation occurred, only about half the samples were collected during this midluteal phase interval. Sera were stored in polypropylene cryovials and stored in the University of Hawaii -18 °C freezer before being shipped to the NIOSH Endocrinology Laboratory and stored at -80 °C until assayed.

All serum samples were analyzed for FSH and LH (immunofluorometric assays, Perkin Elmer, A031-101 and A017-201). Samples from women were also analyzed for estradiol and progesterone (radioimmunoassay, Diagnostic Systems Laboratories, DSL-4400 and DSL-3900). Samples from men were also analyzed for total testosterone (radioimmunoassay, Diagnostic Products Corporation, DCP-TKTT), inhibin-B (enzyme linked immunosorbent assay, DSL, DSL-10-84100), sex hormone binding globulin (immunoradiometric assay, DSL, DSL-7400), and albumin (Vitros 250 Chemical Analyzer, Ortho Clinical Diagnostics). All analyses were performed according to manufacturers' instructions. Free testosterone concentrations were calculated using total testosterone, sex hormone binding globulin, and albumin concentrations and association constants as previously described (McCann and Lirkish, 1985; Vermeulen et al., 1999). The intra-assay coefficients of variation for serum LH, FSH, progesterone, estradiol, testosterone, sex hormone binding globulin, and inhibin B were 7.6%, 8.0%, 8.6%, 4.2%, 6.0%, 5.3%, and 12.4%, respectively; interassay coefficients of variation were 1.0%, 3.2%, 2.5%, 3.9%, 8.9%, 4.0%, and 10.5%, respectively.

2.7. Semen analyses

Male participants were instructed to abstain from sexual behavior involving ejaculation for 3–7 days before collecting fresh semen samples by masturbation into plastic vials. Samples were collected at a Clinical Laboratories of Hawaii, LLP medical laboratory and processed within one hour using standard protocols for measuring volume, viscosity, and sperm concentration, total count, motility and morphology. For dichotomous analyses, low was defined as sperm concentration <20 million/mL, motile sperm $<50\%$, and sperm with normal morphology $<30\%$ (World Health Organization, 1992).

2.8. Statistical methods

We evaluated distributions of endocrine and semen endpoints for normality, i.e., the numerical value for skewness was within ± 2 standard errors of the skew coefficient of the normal distribution. Luteal phase length and total testosterone were the only normally distributed endpoints. All skewed continuous variables were cube root transformed for the regression analyses. We chose the cube root because it is a normalizing transformation that could be applied to variables with negative values (day of luteal transition slope) and variables with values between 0 and 1. Since FSH slope before menses could be positive and negative, this endpoint was not transformed. We did not perform back-transformations.

Because the ages at which various pubertal milestones were achieved are highly correlated with one another, we conducted factor analyses for the male and female pubertal variables using principal component analysis with Varimax rotation, Kaiser normalization, and initial Eigen values of 1 to identify uncorrelated measures of pubertal milestones.

To identify potential confounding variables, we considered covariates that are recognized or potential independent risk factors for the outcomes that also could

Table 1
Characteristics of female (number=183) and male (number=216) participants.

Characteristics	Females Number (%)[a]	Males Number (%)[a]
Ethnicity		
Asian (not Pacific Islander)	51 (27.9)	55 (25.5)
White or White/non-Hawaiian mix	23 (12.6)	46 (21.3)
Filipino	11 (6.0)	15 (6.9)
Hawaiian or Hawaiian mix	63 (34.4)	73 (33.8)
Other	35 (19.1)	27 (12.5)
Employment status		
Employed	127 (69.4)	172 (79.6)
Unemployed	14 (7.7)	22 (10.2)
Student	33 (18.0)	22 (10.2)
Homemaker	9 (4.9)	*0*
Occupational exposures		
Solvents	16 (8.7)	40 (18.5)
Pesticides	1 (0.5)	11 (5.1)
Other Chemicals	15 (8.2)	37 (17.1)
Ionizing Radiation	3 (1.6)	3 (1.4)
Lead	2 (1.1)	8 (3.7)
Personal habits		
Drinks alcohol	167 (91.3)	198 (91.7)
Smokes cigarettes	46 (25.1)	79 (36.6)
Used marijuana in past month	20 (10.9)	58 (26.9)
Maternal pregnancy characteristics[b]		
Home was located near farm(s)	15 (8.2)	27 (12.5)
Pesticide exposure	7 (2.0)	8 (2.2)
Smoked cigarettes	21 (11.5)	33 (15.3)
Drank alcohol	11 (6.0)	18 (8.3)
Stress—moderate or greater	87 (47.5)	67 (31.0)
Breastfed or breast & bottle-fed	115 (62.8)	136 (63.0)
Gynecological history		
Regular predictable cycles	121 (67.2)	NA
Premenstrual syndrome	8 (4.4)	NA
Amenorrhea (no period for >12 months)	18 (9.9)	NA
Irregular cycles	27 (14.9)	NA
Heavy periods	14 (7.7)	NA
Urologic history		
Orchitis	NA	1 (0.5)
Varicocele	NA	2 (0.9)
Hypospadias	NA	2 (0.9)
Cryptorchidism	NA	1 (0.5)
Heptachlor epoxide exposure—mothers' cow milk consumption		
0–5 glasses/week	72 (39.3)	75 (34.7)
6–11 glasses/week	56 (30.6)	53 (24.5)
12–19 glasses/week	25 (13.7)	54 (25.0)
≥ 20 glasses/week	24 (13.1)	30 (13.9)
	Mean (SD)	**Mean (SD)**
Mothers' cow milk consumption (glasses/week)	9.2 (8.5)	10.3 (9.2)
Mothers' age at participant's birth (years)	28.0 (5.4)	27.7 (5.2)

[a] Some of the percentages may not add up to 100% due to missing responses.
[b] Pregnancy with study participant.

be plausibly associated with heptachlor epoxide exposure (mothers' milk consumption during pregnancy in categories of glasses/week) based on the scientific literature and using causal diagrams. We considered some groups of variables shown in Table 1, such as employment status and occupational exposures, not to be potential confounding variables because they were not plausibly associated with maternal cow milk consumption before the participants were born. We did consider personal habits as possible, but unlikely, potential confounding variables because these behaviors could be surrogates for exposure to confounding gestational social factors or toxic exposures that could have led to changes in behaviors (e.g., the way that gestational lead exposure might alter behaviors among adolescents). We considered body mass index (BMI) to be a potential confounding variable with the same reasoning that it could be a surrogate for a confounding gestational exposure to an endocrine disrupting chemical. We considered the group of maternal pregnancy characteristics in Table 1 to be potential confounding variables.

To evaluate the potential confounding variables, we examined the prevalence of the exposures or characteristics in the total Oahu born population, and the pattern and strength of bivariate associations between the variables and heptachlor epoxide exposure based on maternal cow milk consumption. We considered but did not adhere to specific criteria for magnitude of strength of associations between the variables and maternal cow milk consumption to identify variables to be considered as potential confounding variables.

We used linear models to analyze the relationships between mother's milk consumption (0–5 glasses per week, 6–11 glasses per week, and ≥ 12 glasses per week) and the female urinary endocrine endpoints, male semen analysis endpoints, male and female serum hormone concentrations, and male and female pubertal milestone endpoints. We used logistic regression to model the relationship between mother's milk consumption and dichotomous endpoints (ovulatory versus anovulatory menstrual cycles, normal versus low sperm concentration, sperm motility, and sperm morphology). We also tested linear models in which mother's cow milk consumption was modeled as a continuous variable. We included ethnicity in all models because many of the endpoints are affected by ethnicity and maternal cow milk consumption during pregnancy was clearly different by ethnicity. We included all potential confounding variables that changed the regression coefficients for mothers' milk consumption by an appreciable amount (approximately 10%) for at least one outcome variable in any of the adjusted models, with some exceptions described below. In general, adjusting for all of these variables in the models for all outcome variables did not appreciably widen the 95% confidence intervals for the associations with maternal milk consumption compared to the most parsimonious model for each outcome, and we feel that using a consistent set of covariates facilitates comparisons among the different outcomes.

We conducted sensitivity analyses in which non-Oahu-born participants were included in the lowest category of maternal cow milk consumption (0–5 glasses/week) because their mothers would not have been consuming cow milk contaminated with heptachlor epoxide. We also conducted analyses in which participants were stratified by whether or not they were breastfed.

3. Results

3.1. Study participant characteristics

Table 1 summarizes characteristics of the female and male study participants. Among the females, the largest ethnic group was Hawaiian or part-Hawaiian, followed by Asian (Chinese, Japanese, or Korean), other, white or white/non-Hawaiian mix, and Filipino, in descending order. Among the men, the largest ethnic group was Hawaiian or part-Hawaiian, followed by Asian, white or white/non-Hawaiian mix, other, and Filipino, in descending order. About 63% of female and male participants' mothers breastfed or breast and bottle-fed the participant as an infant. About 39%, 31%, 14%, and 13% of the females' mothers recalled drinking 0–5, 6–11, 12–19, or ≥ 20 glasses of cow milk weekly, respectively, during that pregnancy. About 35%, 25%, 25% and 14% of the males' mothers recalled drinking 0–5, 6–11, 12–19 or ≥ 20 glasses of cow milk per week while pregnant.

3.2. Heptachlor epoxide and female reproductive function

Table 2 shows menstrual cycle and urinary hormone characteristics for the women. Of the 183 female participants, 133 had usable urinary hormone and menstrual cycle data. Median menstrual cycle, follicular phase, and luteal phase lengths were 30 days, 17 days, and 13 days, respectively. Approximately 14% of the cycles for this population were anovulatory. Previous studies have reported wide variability in the percentages of menstrual cycles that are anovulatory in young women 20–24 years of age (from 6–36%), with very long and very short cycles being associated with higher rates of anovulation (Harlow and Ephross, 1995). Supplemental Table 2 shows the means, standard deviations, and medians of the menstrual cycle and urinary reproductive hormone variables for the Oahu-born female participants by category of maternal milk consumption.

Variables that were associated with mothers' consumption of cow milk while pregnant with female participants (Table 3) included: ethnicity, participants' smoking and body mass index;

Table 2
Menstrual cycle and urinary hormone endpoints for female participants (number=133, but not all variables could be calculated for every cycle).

Endpoint	Mean	Median	Standard deviation	Range
Cycle length (days)	31.9	30	9.0	18, 81
Follicular phase length (days)	18.4	17	7.3	8, 55
Luteal phase length (days)	13.0	13	2.7	4, 21
Early follicular FSH level (mIU/mg Cr)	4.95	4.60	2.06	0.20, 11.4
Follicular LH:FSH ratio	1.76	1.18	2.27	0.09, 17.8
Early follicular $E_1$3G level (ng/mg Cr)	6.65	5.93	3.63	1.71, 33.4
Follicular Pd3G level (μg/mg Cr)	1.57	1.40	0.91	0.42, 6.67
Preovulatory LH level (mIU/mg Cr)	25.4	20.1	20.1	1.23, 120.1
Steepest E13G:Pd3G slope	−9.37	−7.28	9.46	−48.29, −0.53
Early–midluteal Pd3G rise (μg/mg Cr/day)	2.79	1.94	3.38	0.12, 21.09
Midluteal Pd3G level (μg/mg Cr)	12.1	10.9	7.80	0.61, 39.77
Midluteal $E_1$3G level (ng/mg Cr)	18.4	16.1	9.78	4.68, 60.4
FSH rise before menses (mIU/mg Cr/day)	0.47	0.41	0.59	−1.00, 2.71
	Number	**Percent of valid cycles**		
Anovulatory cycle	18	14.2		

Table 3
Bivariate analyses of the association between demographic, lifestyle, and maternal characteristics and heptachlor epoxide exposure based on mother's milk consumption for female participants.

	Glasses of milk per week		
Characteristic	**0–5 glasses**	**6–11 glasses**	**≥12 glasses**
	Number (%)	**Number (%)**	**Number (%)**
Ethnicity			
Asian	22 (30.6)	17 (30.4)	10 (20.4)
White/White mix	5 (6.9)	11 (19.6)	7 (14.3)
Hawaiian/part-Hawaiian	21 (29.2)	19 (33.9)	21 (42.9)
Other	24 (33.3)	9 (16.1)	11 (22.4)
Participant's habits			
Smokes cigarettes, No	53 (73.6)	43 (76.8)	44 (89.8)
(>15 per month), Yes	19 (26.4)	13 (23.2)	5 (10.2)
Drinks alcohol, 0 to <3	35 (48.6)	26 (46.4)	21 (42.9)
(drinks/month), 3 to <10	25 (34.7)	24 (42.9)	19 (38.8)
≥10	12 (16.7)	6 (10.7)	9 (18.4)
Uses marijuana, No	62 (86.1)	49 (90.7)	43 (89.6)
Yes	10 (13.9)	5 (9.3)	5 (10.4)
Maternal pregnancy characteristics			
Home near farm, No	61 (89.7)	46 (95.8)	41 (87.2)
Yes	7 (10.3)	2 (4.2)	6 (12.8)
Exposed to pesticides, No	51 (100)	35 (89.7)	34 (94.4)
Yes	0 (0)	4 (10.3)	2 (5.6)
Smoked cigarettes, No	61 (89.7)	36 (75.0)	46 (95.8)
Yes	7 (10.3)	12 (25.0)	2 (4.2)
Drank alcohol, No	71 (98.6)	47 (87.0)	46 (93.9)
Yes	1 (1.4)	7 (13.0)	3 (6.1)
Moderate or greater stress, No	30 (45.5)	24 (49.0)	22 (45.8)
Yes	36 (54.5)	25 (51.0)	26 (54.2)
Breastfed or breast+bottle-fed, No	27 (40.3)	12 (25.0)	10 (20.8)
Yes	40 (59.7)	36 (75.0)	38 (79.2)
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Mother's age at participant's birth	27.9 (5.5)	28.2 (5.4)	27.9 (5.4)
Participant's body mass index (kg/m^2)	23.8 (5.7)	26.5 (7.4)	25.1 (5.7)

and mothers' exposure to pesticides and drinking alcohol while pregnant, and breastfeeding or breast and bottle-feeding the participant. Some variables such as maternal exposure to non-heptachlor pesticides had very low prevalence and no apparent association with mothers' cow milk consumption, so they were not included in the final models. We adjusted for all the other potential confounding variables in the final models based on the change in coefficient criterion described in the Section 2.

Table 4 shows the findings of the adjusted linear regression models for female reproductive function outcomes and maternal cow milk consumption. The estimated marginal mean (EMM) is the mean of the outcome variable adjusted for all of the covariates in the model with maternal cow milk consumption at the reference category of 0–5 glasses/week. Based on adjusted linear regression models, only two menstrual cycle endpoints were associated with maternal cow milk consumption (Table 4). Luteal phase length was longer in women whose mothers drank ≥12 glasses/week compared to those who drank 0–5 glasses/week (the difference from the estimated marginal mean of 12.7 was 1.707 with 95% confidence interval (CI): 0.281, 3.231). Noting that the standard deviation of luteal phase length in Table 2 was 2.7 days, this effect is approximately 0.6 standard deviations in this study population. There was a slower drop in the ratio of estrogen to progesterone metabolites at the time of ovulation (steepest $E_1$3G:Pd3G slope) for women whose mothers were in the highest milk consumption group (the difference from the estimated marginal mean of −2.00 was 0.263 with 95% CI: −0.001, 0.528). Because this outcome was cuberoot transformed for the analysis, one can back-transform the findings to interpret the magnitude of the effect. The estimated steepest $E_1$3G:Pd3G slope for women whose mother drank 0–5 glasses/week cow milk was the cube of the estimated marginal mean or $-2.00^3=-8.00$. (This slope is slightly different than the crude mean shown in Table 2 because it is adjusted for the covariates.) The estimated steepest $E_1$3G:Pd3G slope for women who drank≥12 glasses/week cow milk was the cube of the sum of estimated marginal mean and the regression coefficient or $(-2.00+0.263)^3=-5.24$. Therefore, the linear difference was 2.76 or approximately 0.30 standard deviation difference from the mean in this study population. A suggestive association for increased midluteal Pd3G rise was observed in women whose mothers consumed 6–11 glasses of milk per week, but this effect disappeared for the highest maternal milk consumption group. There were no other suggestive associations with the regression coefficients being consistent with null association and estimated effect sizes generally less than 0.20 standard deviations of the study population mean. No apparent associations for any outcomes were found with linear regression analyses when mothers' cow milk consumption was modeled as a continuous variable. Logistic regression showed suggestive trends toward lower prevalence of anovulatory menstrual cycles in participants with increasing maternal milk consumption (*OR*=0.87, 95% CI: 0.21–3.62 for 6–11 glasses/week and OR=0.54, 95% CI: 0.11–2.59 for ≥12 glasses/week, relative to 0–5 glasses/week). Adjusted linear regression models for serum estradiol, progesterone, LH, and FSH for the subset of 58 women whose blood was drawn during the midluteal

Table 4
Adjusted[a] linear models of the effects of heptachlor exposure (maternal cow milk consumption while pregnant) on reproductive function of female participants.

Endpoint (number of women)	Estimated marginal mean [b]	Unstandardized coefficient (95% confidence interval)	
		6–11 glasses/week[c]	≥12 glasses/week[c]
Cycle length (108)	3.12	0.028 (−0.085, 0.142)	−0.010 (−0.124, 0.103)
Follicular phase length (104)	2.56	−0.037 (−0.186, 0.113)	−0.091 (−0.237, 0.055)
Luteal phase length (98)	12.7	0.947 (−0.521, 2.415)	1.707 (0.281, 3.132)
Early follicular FSH level (119)	1.69	−0.054 (−0.170, −0.062)	−0.077 (−0.192, 0.037)
Follicular LH: FSH ratio (114)	1.21	0.050 (−0.098, 0.197)	−0.057 (−0.202, 0.089)
Early follicular $E_1$3G level (119)	1.98	0.043 (−0.072, 0.158)	0.045 (−0.068, 0.159)
Follicular Pd3G level (118)	1.18	−0.017 (−0.104, 0.069)	0.048 (−0.038, 0.133)
Preovulatory LH level (101)	3.12	0.201 (−0.170, 0.571)	−0.267 (−0.632, 0.097)
Steepest DLT slope (90)	−2.00	−0.050 (−0.325, 0.224)	0.263 (−0.001, 0.528)
Early–midluteal Pd3G rise (84)	1.14	0.211 (−0.020, 0.442)	0.006 (−0.231, 0.243)
Midluteal Pd3G level (96)	2.28	0.092 (−0.179, 0.363)	0.019 (−0.256, 0.293)
Midluteal $E_1$3G level (96)	2.61	0.071 (−0.159, 0.301)	0.092 (−0.141, 0.325)
FSH rise before menses (99)	0.574	0.043 (−0.290,0.375)	0.166 (−0.160, 0.491)

[a] Adjusted for participant's ethnicity, smoking more than 15 cigarettes past month, BMI, mother's alcohol use while pregnant, and mother breastfed participant. All analyses were carried out on the cube root transformed endpoints, except 'luteal phase length' and 'FSH rise before menses' were not transformed. Back-transformations were not done.

[b] Estimated marginal mean (EMM) is the mean of the outcome adjusted for all covariates with maternal cow milk consumption at the reference category—based on a general linear model. The coefficients are the change from the EMM for the categories of maternal cow milk consumption.

[c] Reference category is 0–5 glasses/week.

phase did not reveal any apparent associations with mothers' cow milk consumption (data not shown).

We conducted sensitivity analyses that included non-Oahu-born participants in the lowest category of maternal milk consumption, i.e. equivalent to consuming none of the contaminated milk on Oahu. The results of these analyses did not differ appreciably from the analyses presented. We also conducted analyses stratified by maternal breastfeeding of the participants. For most outcomes, the direction of the association between maternal milk consumption and the outcome was similar in breastfed and non-breastfed participants (not shown). These analyses were limited by the small numbers of participants in each stratum. However, for cycle length, steepest $E_1$3G:Pd3G slope, and follicular Pd3G level, the associations were positive for the breastfed participants and negative for the non-breastfed participants. For midluteal Pd3G rise, the associations were negative for the breastfed participants and positive for the non-breastfed participants.

3.3. Heptachlor epoxide and male reproductive function

Table 5 shows semen and endocrine endpoints for the men. Mean sperm parameters were within normal values (World Health Organization, 1992). However, 19.8% of the samples analyzed were below the reference level of 20 million sperm per milliliter; 17.8% were below the reference level of 40 million total sperm; 10.7% were below the reference level of 50% motile sperm, and 14.5% were below the reference level of 30% of sperm with normal morphology. Mean serum LH, FSH, testosterone, and inhibin B values were within expected ranges provided by the assay kit manufacturers; however, several individuals had hormone levels above or below the expected ranges. 6.7% of men had total testosterone concentrations below the expected lower limit of 2.5 ng/ml; 7.7% had inhibin B concentrations below the expected lower limit of 31 pg/ml; 0.5% had LH and FSH concentrations below the expected lower limit of 1 mIU/ml; 4.2% had LH concentrations greater than the expected upper limit of 8.4 mIU/ml; 1.4% had FSH concentrations greater than the expected upper limit of 10.5 mIU/ml. The one individual with low concentrations of both LH and FSH had oligospermia and a normal testosterone concentration. Two individuals with high serum FSH and normal LH had low inhibin B, consistent with Sertoli cell dysfunction; one of these had oligospermia and one had high sperm counts.

Table 5
Sperm and serum hormone endpoints for male participants.

Endpoint	Mean	Median	Standard deviation	Range
Sperm analyses (N=171)				
Concentration (million/ml)	76.1	55	73.8	0, 449
Count (million/ejaculate)	229.2	152	241.4	0, 1347
Motility (% motile)	74.1	75	18.7	14, 100
Morphology (% normal)	51.4	52	18.8	12, 100
Serum (N=212)				
Total testosterone (ng/ml)	4.2	4.1	1.4	1.2, 9.6
Free testosterone (pg/ml)	88.1	80.6	34.8	19, 254
SHBG (nmol/L)	30.6	28.8	15.9	2, 85
Inhibin B (pg/ml)	97.6	88.9	53.5	12, 289
FSH (mIU/ml)	3.7	3.2	2.0	0.03, 13.7
LH (mIU/ml)	4.6	4.1	2.0	0.03, 14.6

Three individuals with high LH concentrations had low testosterone concentrations and normal inhibin concentrations; one of these had a normal and two a high FSH concentration; one had azoospermia and two had normospermia. Supplemental Table 3 shows the means, SDs, and medians of the semen parameters and serum reproductive hormone variables for the Oahu born male participants by category of maternal cow milk consumption.

Two cases of hypospadias were reported by Oahu-born men whose mothers consumed 5 and 14 glasses of milk weekly, respectively. One cryptorchidism case was reported by a man whose mother consumed 14 glasses of milk weekly. These small numbers of cases precluded testing for associations to heptachlor exposure.

Of the covariates considered for the analysis, the participant's ethnicity and alcohol use and mothers' breast- or breast- and bottle-feeding of the participant were considered to be potential confounders based on their association with heptachlor epoxide exposure (Table 6) and the change in coefficient criterion described in Section 2.

Adjusted linear models revealed no consistent associations for semen or endocrine endpoints with mothers' cow milk consumption, indeed for many outcomes the directionality of the coefficients

Table 6
Bivariate analyses of the association between demographic, lifestyle, and maternal characteristics and heptachlor epoxide exposure based on mothers' milk consumption for male participants.

Characteristics	Glasses of milk per week		
	0–5 glasses Number (%)	6–11 glasses Number (%)	≥12 glasses Number (%)
Ethnicity			
Asian	21 (28.0)	12 (22.6)	22 (26.2)
White/ White-mix	14 (18.7)	19 (35.8)	12 (14.3)
Hawaiian/part-Hawaiian	19 (25.3)	17 (32.1)	34 (40.5)
Other	21 (28.0)	5 (9.4)	16 (19.1)
Participant's habits			
Smokes cigarettes, No	45 (60.0)	34 (64.2)	56 (66.7)
>(15 per month), Yes	30 (40.0)	19 (35.8)	28 (33.3)
Drinks alcohol, 0 to <3	18 (24.3)	13 (24.5)	32 (38.1)
(drinks/month), 3 to <10	32 (43.2)	22 (41.5)	28 (33.3)
≥10	24 (32.4)	18 (34.0)	24 (28.6)
Uses marijuana, No	52 (72.2)	35 (67.3)	62 (77.5)
Yes	20 (27.8)	17 (32.7)	18 (22.5)
Maternal pregnancy characteristics			
Home near farm, No	60 (85.7)	37 (94.9)	67 (81.7)
Yes	10 (14.3)	2 (5.1)	15 (18.3)
Exposed to pesticides, No	68 (97.1)	39 (97.5)	79 (95.2)
Yes	2 (2.9)	1 (2.5)	4 (4.8)
Smoked cigarettes, No	58 (82.9)	32 (80.0)	70 (84.3)
Yes	12 (17.1)	8 (20.0)	13 (15.7)
Drank alcohol, No	68 (90.7)	49 (92.5)	78 (92.9)
Yes	7 (9.3)	4 (7.5)	6 (7.1)
Moderate or greater stress, No	45 (65.2)	25 (64.1)	55 (66.3)
Yes	24 (34.8)	14 (35.9)	28 (33.7)
Breastfed or breast+bottle-fed, No	26 (37.1)	9 (22.5)	22 (26.5)
Yes	44 (62.9)	31 (77.5)	61 (73.5)
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Mother's age at participant's birth	27.7 (4.9)	27.1 (5.0)	27.8 (5.5)
Participant's body mass index (kg/m^2)	26.5 (5.7)	27.4 (6.6)	27.0 (6.2)
Abstinence before semen sample (days)	4.0 (1.4)	3.9 (2.3)	4.1 (1.3)

Table 7
Adjusted[a] linear models of the effects of heptachlor exposure (mothers' cow milk consumption while pregnant) on reproductive function of male participants.

Endpoint	Estimated marginal mean[b]	Unstandardized coefficient (95% confidence interval)	
		6–11 glasses/week[c]	≥12 glasses/week[c]
Sperm characteristics (N)			
Concentration (162)	3.849	−0.078 (−0.681, 0.525)	0.016 (−0.486, 0.517)
Count (162)	5.346	0.031 (−0.888, 0.950)	0.197 (−0.568, 0.962)
Motility (161)	0.992	−0.008 (−0.109, 0.094)	0.028 (−0.056, 0.112)
Morphology (159)	0.790	0.004 (−0.090, 0.099)	−0.029 (−0.108, 0.049)
Serum hormones (N)			
Total testosterone (188)	4.006	0.178 (−0.393, 0.750)	−0.030 (−0.495, 0.436)
Free testosterone (188)	4.424	−0.042 (−0.277, 0.194)	−0.079 (−0.271, 0.113)
SHBG (188)	2.871	0.143 (−0.094, 0.380)	0.087 (−0.106, 0.280)
Inhibin B (187)	4.426	−0.073 (−0.437, 0.291)	0.026 (−0.268, 0.321)
LH (189)	1.745	−0.052 (−0.149, 0.045)	−0.061 (−0.140, 0.017)
FSH (189)	1.590	−0.086 (−0.195, 0.023)	−0.043 (−0.132, 0.046)

[a] Adjusted for alcohol consumption, ethnicity, and mother's breast or breast and bottle-feeding. All analyses were performed using the cube root transformed variables, except total testosterone was not transformed. Back transformations were not done.
[b] Estimated marginal mean (EMM) is the mean of the outcome adjusted for all covariates with maternal cow milk consumption at the reference category—based on a general linear model. The coefficients are the change from the EMM for the categories of maternal cow milk consumption and percent motile sperm and percent normal morphology were arcsine square root transformed.
[c] Reference category is 0–5 glasses/week.

were opposite for the two categories of mothers' cow milk consumption (Table 7). While FSH and LH levels tended to be lower in men whose mothers drank 6–11 glasses/week and ≥12 glasses/week of milk, these associations were weak and the confidence intervals encompassed the null value. Number of days of sexual abstinence before semen sample collection, a strong predictor of sperm concentration and total count, was available for 68% (117/171) of men who provided samples. When we modeled sperm

parameters to include number of days of abstinence as a covariate, coefficient estimates changed substantially in size and some changed in direction, but again no consistent effects of mothers' milk consumption were observed (not shown). When linear regression analyses modeled milk consumption as a continuous variable (with or without abstinence), none of the sperm or endocrine parameters was associated with milk consumption (not shown). Nor was milk consumption associated with dichotomized sperm characteristics when analyzed by logistic regression (Supplemental Table 4).

We conducted sensitivity analyses that included non-Oahu-born participants in the lowest category of maternal milk consumption. The results of these analyses did not differ appreciably from the analyses presented. We also conducted analyses stratified by maternal breastfeeding of the participants. These analyses were limited by the small numbers of participants in each stratum. For some outcomes, the direction of the association between maternal milk consumption and the outcome was similar in breastfed and non-breastfed participants. However, for sperm concentration and total sperm count, the associations with maternal milk consumption of 6–11 glasses per week were positive in the non-breastfed participants and negative in the breastfed participants, while the associations with maternal milk consumption of ≥ 12 glasses per week were essentially null; sperm count was associated with mothers' cow milk consumption in the non-breastfed participants whose mothers drank 6–11 glasses of milk per week (unstandardized coefficient = 1.735 95% CI: 0.099, 3.371). Among men who were not breastfed, free testosterone (unstandardized coefficient = −0.297, 95% CI: −0.669, 0.076), total testosterone (unstandardized coefficient = −0.605 95% CI: −1.422, 0.213), and inhibin B (unstandardized coefficient = −0.239, 95% CI: −0.778, 0.299) concentrations showed negative associations with maternal milk consumption of ≥ 12 glasses per week, as well as lesser negative associations with maternal milk consumption of 6–11 glasses per week. For these outcome variables, the associations were essentially null or somewhat positive in men who were breastfed.

Table 8
Self-reported ages (mean ± standard deviation) of onset for pubertal milestones in study participants, arranged by maternal milk consumption.

	Glasses per week		
	0–5	6–11	≥ 12
Female participants ($N=183$)			
Age axillary hair appeared	12.3 ± 1.5	12.2 ± 1.6	12.1 ± 2.0
Age hair on legs appeared	11.9 ± 1.6	12.0 ± 1.6	11.9 ± 1.9
Age pubic hair appeared	11.9 ± 1.3	12.2 ± 1.5	12.0 ± 1.8
Age breast development began	11.7 ± 1.6	11.8 ± 2.0	11.7 ± 2.0
Age at menarche	12.4 ± 1.5	12.1 ± 1.5	12.6 ± 1.5
Male participants ($N=212$)			
Age axillary hair appeared	13.1 ± 1.6	12.8 ± 1.4	13.5 ± 1.5
Age hair on legs appeared	13.3 ± 1.8	12.7 ± 1.9	13.3 ± 2.0
Age pubic hair appeared	12.9 ± 1.4	12.5 ± 1.3	13.1 ± 1.5
Age voice changed	13.7 ± 1.6	13.2 ± 1.5	13.9 ± 1.9
Age started shaving	16.5 ± 1.9	16.0 ± 2.2	16.5 ± 2.0
Age at first ejaculation	13.8 ± 2.3	13.8 ± 2.6	13.4 ± 1.9

3.4. Age of pubertal milestones

For male participants, age of onset for all but one pubertal milestone followed a slight U-shaped pattern with mothers' cow milk consumption; youngest ages for most milestones occurred in participants whose mothers drank intermediate amounts of milk (Table 8). Linear regression models for the pubertal milestones were adjusted for participant's ethnicity, maternal cigarette smoking and alcohol use during pregnancy with the participant, and breastfeeding of the participant; the models were not adjusted for current participant habits because we did not collect information about the age of onset of these habits. The adjusted models revealed no apparent effects of mothers' cow milk consumption for male or female participants (Table 9). There was a suggestive trend towards older age when the male participants first noticed hair in the axillae with maternal milk consumption of ≥ 12 glasses/week compared to 0–5 glasses/week. This association persisted and there was also a suggestive positive association with the age at which pubic hair growth was first noted when we included non-Oahu-born participants in the reference category of 0–5 glasses/week of maternal milk consumption. In analyses that did and did not include non-Oahu-born women in the reference maternal milk consumption category, we observed weak associations (unstandardized coefficient = −0.381, 95% CI: −0.964, 0.201; unstandardized coefficient = −0.460, 95% CI: −0.994, 0.075, respectively) for earlier age at menarche in daughters whose mothers consumed 6–11 glasses/week compared to 0–5 glasses per week, but there was no evidence for a

Table 9
Adjusted[a] linear regression models of the effects of maternal milk consumption on self-reported ages of pubertal milestones.

	Estimate marginal mean[b]	Unstandardized coefficient (95% confidence interval)	
		6–11 glasses per week[c]	≥ 12 glasses per week[c]
Females *(number)*			
Axillary hair (160)	12.189	−0.153 (−0.804, 0.498)	−0.021 (−0.654, 0.613)
Hair on legs (159)	11.922	−0.223 (−0.870, 0.424)	−0.051 (−0.675, 0.573)
Pubic hair (160)	11.759	−0.081 (−0.495, 0.656)	0.140 (−0.420, 0.700)
Breast development (160)	11.587	−0.015 (−0.707, 0.676)	0.074 (−0.599, 0.747)
Menarche (160)	12.410	−0.381 (−0.964, 0.201)	0.194 (−0.377, 0.765)
Males *(N)*			
Axillary hair (193)	12.902	−0.026 (−0.610, 0.559)	0.439 (−0.032, 0.910)
Hair on legs (192)	13.862	−0.585 (−1.356, 0.186)	0.054 (−0.570, 0.678)
Pubic hair (193)	12.939	0.020 (−0.541, 0.580)	0.339 (−0.113, 0.791)
Voice changed (193)	13.761	−0.322 (−0.963, 0.318)	0.304 (−0.212, 0.820)
Started shaving (193)	16.953	−0.573 (−1.361, 0.215)	−0.103 (−0.737, 0.532)
First ejaculation (192)	13.395	0.166 (−0.729, 1.061)	−0.345 (−1.068, 0.377)

[a] Adjusted for participant's ethnicity, mother's alcohol consumption and smoking while pregnant and breastfeeding of participant.
[b] Estimated marginal mean (EMM) is the mean of the outcome adjusted for all covariates with maternal cow milk consumption at the reference category—based on a general linear model. The coefficients are the change from the EMM for the categories of maternal cow milk consumption.
[c] Reference category is 0–5 glasses per week.

dose response, as the effect disappeared for daughters whose mothers drank ≥12 glasses/week.

To further evaluate the puberty milestone outcomes and to address the high correlation among these outcomes, we also conducted factor analyses. These factor analyses identified one component for males, which loaded on the hair growth variables and explained 52.7% of the variance, and two components for females, one which loaded on the hair growth and breast development variables and explained 57.3% of the variance and one which loaded on the menarche variable and explained 25.6% of the variance. Based on the factor analyses, we generated new variables by calculating the mean age for the growth of axillary, leg, and pubic hair in the men and the mean age for hair growth plus breast development in the women. Regression analyses using these new variables as endpoints revealed no significant associations with maternal milk consumption during pregnancy (not shown).

For males and females, stratifying analyses by whether or not the participant was breastfed revealed negative associations of ages at which pubertal milestones were achieved with maternal milk consumption among those not breastfed and positive associations among those who were breastfed; however, few of the associations were statistically significant. We observed an earlier age at menarche among women who were not breastfed and whose mothers drank 6–11 glasses/week compared to those whose mothers drank 0–5 glasses/week (unstandardized coefficient = −1.027, 95% CI: −2.008, −0.046). Among the men, we observed an increase in the ages at which axillary (unstandardized coefficient = 0.699, 95% CI: 0.140, 1.258) and pubic (unstandardized coefficient = 0.722, 95% CI: 0.216, 1.228) hair were first noted among men who were breastfed and whose mothers drank ≥12 glasses/week compared to those whose mothers drank 0–5 glasses/week. In contrast, we observed trends towards earlier ages at which axillary hair (unstandardized coefficient = −1.126, 95% CI: −2.436, 0.004) and pubic hair (unstandardized coefficient = −1.108, 95% CI: −2.393, 0.178) were first noted among men who were not breastfed and whose mothers drank 6–11 glasses/week compared to those whose mothers drank 0–5 glasses/week.

4. Discussion

The results of this study provide limited evidence for an association between gestational and/or lactational exposure to heptachlor epoxide and deficits in reproductive function for young adult men and women. We observed few apparent associations between mothers' cow milk consumption during pregnancy – a surrogate for heptachlor epoxide exposure – and reproductive function in the male and female offspring. For women, maternal milk consumption was directly associated with longer luteal phase and with slower $E_1$3G:Pd3G drop after ovulation. For men, we observed suggestive inverse associations between maternal milk consumption and serum FSH and LH concentrations.

Menstrual cycle characteristics of our study participants, including cycle length and proportion of anovulatory cycles, were similar to those previously reported (Harlow and Ephross, 1995; Wilcox et al., 1995). The median menstrual cycle length was reportedly 28 days for 20–24 year old women in two studies; the range was 20–39 days (Harlow and Ephross, 1995). Wide variability has also been observed in the percentages of menstrual cycles that are anovulatory in young women aged 20–24 (ranging from 6–36% of cycles), with very long and very short cycles being associated with higher rates of anovulation (Harlow and Ephross, 1995). On the other hand, semen parameter values in our study were slightly higher than those reported for fertile men (Bonde et al., 1998; Guzick et al., 2001). The difference is probably due to our younger subjects (Kidd et al., 2001).

The biological significance of the association of heptachlor epoxide exposure with luteal phase length and $E_1$3G:Pd3G slope after ovulation is uncertain. Mean luteal phase length for all categories of mothers' cow milk consumption was within the normal 12–14 day range (Lenton et al., 1984; Miller and Soules, 2009). We also did not observe evidence of a greater proportion of women with unusually long luteal phases (>2 SD above our population mean) in the groups with higher maternal cow milk consumption (data not shown). The slower drop in the $E_1$3G:Pd3G ratio after ovulation, reflects sluggish functional (endocrine secretion) transition from the Graafian follicle to the corpus luteum, and may predict less fertile cycles (Baird et al., 1997). However, in the current study, we did not detect associations between exposure and other luteal phase variables associated with reduced conception rates, including midluteal Pd3G and $E_1$3G levels (Baird et al., 1999; Baird et al., 1997). In fact, there was a suggestive trend towards increased midluteal Pd3G in women whose mothers drank 6–11 glasses of milk per week compared to those whose mothers drank 0–5 glasses per week (Table 4). Higher midluteal Pd3G has been associated with increased prevalence of conceptional menstrual cycles (Baird et al., 1997).

The marginal associations we detected between mothers' cow milk consumption and LH and FSH levels in men did not show evidence of a dose-response with increasing exposure. Furthermore, mothers' cow milk consumption, when analyzed as a continuous variable, was also not associated with FSH or LH concentration ($P > 0.11$). The absence of linear dose-responses for LH and FSH, coupled with the absence of apparent associations between exposure and other endocrine and semen parameters tempers our confidence that these marginal observed associations represent meaningful effects of developmental exposure to heptachlor epoxide.

Previous studies of associations between prenatal organohalogen exposures and pubertal milestones have reported varied results. Menarche and pubic hair, but not breast development, appeared earlier in breastfed girls exposed to high polybrominated biphenyl levels *in utero*, than those with low exposure *in utero* or those not breastfed (Blanck et al., 2000). Prenatal exposure to persistent organochlorine pollutants via maternal fish consumption was not associated with age at menarche (Axmon, 2006). A trend towards younger menarchial age with increasing maternal serum DDE levels during pregnancy disappeared after adjusting for pubertal body size (Vasiliu et al., 2004). Peripubertal exposure to dioxins and PCBs has been associated with later onset of puberty in males (Den Hond and Schoeters, 2006; Korrick et al., 2011). We did not observe statistically significant effects of maternal milk consumption on age at any pubertal milestone in either gender, nor did we observe significant effects of maternal milk consumption on composite variables based on factor analyses for each gender. We conducted additional sensitivity analyses examining whether maternal milk consumption was associated with very early age of the composite puberty variables (< 2 SD below the population mean) or late age of these variables (>2 SD above the population means). These analyses did not reveal any evidence that exposure to heptachlor epoxide via maternal milk consumption during pregnancy and lactation affected the tails of the distributions of the ages at which pubertal milestones were attained in the males (analyses not shown). However, we observed that 8.2% of the women whose mothers drank ≥12 glasses of milk/week fell into the very early composite puberty age group, compared to only 1.4% and 1.9%, respectively, of the women whose mothers drank 0–5 or 6–11 glasses of milk per week, respectively (analyses not

shown and effect of milk consumption not statistically significant). We did not observe such a pattern for age at menarche.

Finally, we observed apparent effect modification by maternal breastfeeding of the participant on male age at puberty, with ages at axillary, pubic, and leg hair growth onset decreasing with maternal milk consumption among men who were not breastfed and increasing with maternal milk consumption among men who were breastfed. Previous studies have reported later age of menarche in daughters who were exclusively breastfed (Minami et al., 2000) and earlier onset of menarche in daughters who were fed formula (Novotny et al., 2003), but we are not aware of studies that examined the effects of breastfeeding on male puberty. The importance of our observation is uncertain, but may indicate competing effects of breastfeeding alone and developmental heptachlor exposure on male puberty.

Cryptorchidism in humans may be associated with exposure to persistent organochlorine compounds. Concentrations of 17 of 21 organochlorine pesticides or their metabolites in breast milk were higher for cryptorchid cases than controls (Damgaard et al., 2006). Concentrations of the eight most abundant insecticide metabolites, including heptachlor epoxide, were associated with cryptorchidism as a group, but only concentrations of trans-chlordane were individually associated with cryptorchidism (Damgaard et al., 2006). Higher serum heptachlor epoxide levels in third trimester women were not associated with risk of cryptorchidism in their sons (Pierik et al., 2007), although odds ratios were >1 for six of seven persistent organochlorine insecticides measured, including oxychlordane and heptachlor epoxide. Levels of heptachlor epoxide in fat were associated with cryptorchidism in a study of 18 cases and 30 controls (Hosie et al., 2000). In the present study, we encountered one cryptorchid case and two hypospadias cases among 216 men. These rates are similar to those for reference populations (National Birth Defects Prevention Network, 2005). The small number of observations precludes meaningful analyses.

A limitation of the present study is the lack of direct measurements of heptachlor epoxide in the participants or their mothers at the time of the participants' gestation and early infancy, so the only available surrogate measure of exposure was retrospective assessment of mothers' cow milk consumption more than twenty years after the pregnancy. We did not measure serum heptachlor epoxide at the time the participants were recruited for the present study because these levels would not have reflected their exposures during gestation and lactation. We observed some digit preference in the mothers' reported milk consumption during pregnancy, which may reflect the level of inaccuracy for the mothers' recall of milk consumption. Both the retrospective assessment and digit preference would have resulted in non-differential exposure misclassification and biased our results towards the null, limiting our ability to detect a relation of milk consumption to the outcomes. However, we note that the identical questions about past milk consumption used in this study were shown in a previous study eight to nine years after the milk contamination to be highly associated with serum and breast milk concentrations of heptachlor epoxide in adult populations on Oahu.

Another potential limitation is our low recruitment rate. Our sampling frame was the eligible population for an earlier study, which had been completed several years before the current study was initiated. Therefore, we were unable to locate many of these potential participants. Of those we contacted, about one-third declined to participate, one-third were not eligible, and one-third were recruited. The primary reasons individuals were not eligible were because they had moved away from Oahu to the mainland USA or because females were using hormonal contraceptives or had recently been pregnant or breastfeeding. There is no reason to believe that past milk consumption by the participants' mothers would be associated with decisions to move to the mainland USA after graduating high school, so there should be no selection bias due to the loss of these potential participants. Eliminating females who were currently or recently pregnant could potentially have led to a selection bias because these females with proven fertile reproductive function were not included. However, we did not observe clear associations with adverse reproductive outcomes in females, so this type of selection bias is not of concern.

Strengths of the study include use of sensitive markers of reproductive function in men and women and detailed information on important potential confounding variables.

In summary, the present study provides limited indications of disrupted reproductive function in young men and women exposed to heptachlor epoxide during gestation and breastfeeding. While we detected associations between heptachlor epoxide exposure and longer luteal phase length and evidence of a sluggish endocrine transition from the Graafian follicle to the corpus luteum after ovulation, other indicators of luteal function were not associated with heptachlor exposure. Our observation of possible modification by breastfeeding of the effect of prenatal heptachlor exposure on the age at onset of several markers of male puberty is interesting and deserves further study. Taken together, these study results do not provide evidence that men and women suffered clinically significant disturbances of reproductive function due to potential perinatal exposures to heptachlor epoxide from the 1980–1982 milk-contamination on Oahu.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2012.11.001.

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