EFFECTS OF CAFFEINATED BEVERAGES ON REPRODUCTIVE HORMONES IN THE BIOCYCLE STUDY

by

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ABSTRACT

Caffeinated and fructose-rich beverages are widely consumed among women of reproductive age but their association with reproductive hormones is not well understood, due in part to inadequate exposure assessment. Our objectives were to 1) assess the relationship between caffeine and fructose intake and reproductive hormones in healthy premenopausal women, evaluating potential effect modification by race; and 2) determine the validity of the Food Frequency Questionnaire (FFQ) for measuring monthly caffeinated beverage intake compared to multiple 24-hour dietary recalls (24HDR). The BioCycle Study (2005-2007) prospective cohort (*n*=259) included women, ages 18-44, who were followed for 2 menstrual cycles, providing fasting blood specimens at up to 8 visits per cycle, 4 24HDRs per cycle, and an FFQ at the end of each cycle.

Caffeine intake ≥200 mg/day was inversely associated with free estradiol (E2) concentrations among white women (β=-0.15 [95% confidence interval (CI): -0.26, -0.05] and positively associated among Asian women (β=0.61 [95% CI: 0.31, 0.92] after taking into account potential confounders. Women who consumed more added sugar than an average American woman (≥ 73.2 grams/day) or above the 66th percentile in fructose intake (≥ 41.5 grams/day) had elevated free E2 concentrations compared to women who consumed less. Women who consumed ≥1 cup/day of sweetened soda had elevated free E2 (β=0.15 [95% CI: 0.06, 0.24]. Neither artificially sweetened soda intake nor fruit juice

intake ≥1 cup/day was significantly associated with reproductive hormones. Caffeine intake reported in the FFQ was greater than that reported in the 24HDRs (mean=114.1 versus 92.6 mg/day; P=0.006) despite high correlation (r=0.80, P<0.001) and moderate agreement (kappa=0.56, 95% CI: 0.42-0.70).

In summary, moderate caffeine consumption was associated with reduced E2 among white women and elevated E2 among Asian women. Added sugars, total fructose, and sweetened soda were associated with elevated E2 among all races. Further research on the association between caffeine, caffeinated beverage components and reproductive hormones, and whether these relationships differ by race, is warranted. Additionally, although caffeine exposures are highly correlated, absolute intakes differ significantly between measurement tools, highlighting the importance of considering potential misclassification of caffeine exposure when conducting women's health epidemiologic studies.

To Alan, my husband, and Monica Ruth, my daughter, who encouraged and supported me to pursue my dream, despite the many hours and summers away.

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CHAPTER 1

INTRODUCTION

Caffeine intake by women of childbearing age is common in the United States (US). Approximately 89% of reproductive-age women consume caffeine daily, averaging 166 mg/day. This equates roughly to 1.5 to 2 cups of caffeinated coffee depending on the roast and brewing method (1, 2). Only limited research has been conducted on the effect of caffeinated beverages on reproductive hormone levels (3-6) and ovulatory function (7-9) among premenopausal women, and the results have been inconclusive (3-9). Inconsistent results are likely due to methodological limitations, specifically inadequate hormone assessment using standard methods to time a woman's cycle phase; failure to use a validated and reliable caffeine exposure tool; inadequate control for confounding variables; and retrospective study designs that are prone to recall bias. Furthermore, previous studies that have set out to investigate the effect of caffeine on female reproductive hormones and ovulatory function have found effects to differ based on beverage type, indicating that other or additional compounds in beverages besides caffeine may also play a role (3, 4, 6, 9). Abnormal levels of reproductive hormones during the premenopausal years may not only affect ovulatory function in the short term (9), but may also affect the etiology of certain diseases, such as breast cancer, in the long term (10). A clearer understanding of these associations, if any, can inform the

Development of guidelines regarding appropriate consumption of caffeinated beverages for premenopausal women (10).

Mechanisms of Effect on Reproductive Hormones and Ovulatory Function

A variety of pathophysiological effects of caffeine and components of caffeinated beverages on reproductive hormones and ovulatory function have been proposed. Caffeine may enhance steroid production via inhibition of the enzyme phosphodiesterase (4, 11) or alternatively may interfere with estrogen metabolism via inhibition of aromatase, a member of the cytochrome P450 (CYP) superfamily (6, 13). Caffeine may have a protective effect on ovulatory function by lowering leptin levels (12) and improving (i.e., elevating) insulin sensitivity (4, 13, 14). Alternatively, caffeine may interfere with oocyte maturation and thus adversely affect ovulatory function via the phosphodiesterase inhibiting mechanism. Regarding caffeinated beverages, coffee and tea are known to contain antioxidants that may adversely affect ovulation (15) as might certain other components in sodas (9, 11).

Both genetic and environmental factors play a role in caffeine metabolism and evidence has shown that caffeine and caffeine metabolites (including paraxanthine, theophylline, and theobromine) have differing effects on health outcomes (16). Genotypes for cytochrome P-450 1A2 (CYP1A2), the enzyme responsible for caffeine metabolism, vary across ethnic groups, with Asian and African populations appearing to metabolize caffeine more slowly than Caucasians (17, 18). Additionally, the rate of caffeine absorption is slowed by the presence of food in the stomach and the use of oral contraceptives, while the rate of caffeine metabolism nearly doubles for smokers (16, 19).

Effects of Caffeine and Caffeinated Beverages on Female Hormones

Caffeine and Coffee

Since coffee is the largest contributor to caffeine exposure (e.g., 1 cup of coffee has approximately 100 mg of caffeine compared to a cup of tea or soda, which has half to a quarter the caffeine content), most studies find similar results for the effects of caffeine and caffeinated coffee on health outcomes. Research thus far regarding caffeine and coffee intake and female reproductive hormones and ovulatory function is limited and conflicting. Kotsopoulos et al. examined free estradiol (E2) plasma concentrations in 524 predominately white (20) premenopausal women from the Nurses' Health Study (NHS) and follow-up NHSII and found an inverse association in trend analyses for both caffeine and coffee intake and geometric mean levels of luteal (but not follicular) free E2 (6). Similarly, London et al. found an inverse relationship with caffeine and follicular percent free E2 among 325 healthy, premenopausal women (race not specified) from the Massachusetts Women's Health Study (21). In contrast to the above studies, Lucero et al. found coffee and caffeine intake associated in trend analyses with increasing geometric mean levels of early follicular phase total E2 concentrations in a study of 498 predominately (97%) white women ages 36 to 45 (4) while Kinney et al. found no association between caffeine and early follicular phase total E2 concentrations in a study of 188 predominately (95%) white women (mean age=34.0) (5). A study among a small sample (n=50) of Japanese college women found adjusted total caffeine and coffee to be highly correlated to Sex Hormone Binding Globulin (SHBG) concentrations, a glycoprotein that binds to estradiol (3).

Tea

The effects of major types of tea on reproductive hormones have also received limited investigation. One study, which was restricted to Asian women, differentiated exposure by tea type (black, oolong, and green) and found green tea to be inversely correlated with follicular estradiol (3). Animal research supports increased estradiol concentrations for intake of green tea (22). Further research with regard to the effects of black versus green tea on reproductive hormones is justified since previous research has shown differing effects on reproductive cancers (23).

Soda

Only 1 study has investigated soda and premenopausal reproductive hormone levels finding no effect, but restricted their investigation to cola and as explained above, had a small sample size (n=50) and limited exposure/outcome assessment (3). While limited data exist on the effect of soda on reproductive hormones, studies have demonstrated that sodas are associated with impaired fasting glucose and metabolic syndrome (24, 25). Among normal-weight women, increased consumption of fructose-sweetened beverages has been shown to lower circulating concentrations of glucose, insulin and leptin and increase postprandial triacylglycerol concentration (26), all of which influence the feedback mechanisms of reproductive hormones (27, 28).

Effects of Caffeine and Caffeinated Beverages on Ovulatory Function

Only 3 previous studies have investigated the effect of caffeine and/or caffeinated beverages on ovulatory function. To date no association between caffeine intake and

ovulatory function has been found (7-9); soda consumption, however, has been shown to be a risk factor for ovulatory disorder infertility (RR: 1.47, P for trend 0.01) based on data from the NHSII (9). In support of this finding, a previous study looking at effects of caffeinated beverages on fecundability found intake of 1 8-oz serving of caffeinated soda was associated with a 50% reduction in the monthly probability of conception (29). Since soda intake has risen dramatically in the last few decades, rising from approximately 2 8-oz servings/week in 1942 to 2 8-oz servings/day in 2000 (30), and is responsible for the majority of fructose intake among women in the US, many investigators examining the rise of obesity, metabolic syndrome and type 2 diabetes have turned their attention to fructose. While some studies have found adverse health effects from sweetened soda intake (31), other studies have found effects with all types of sodas (irrespective of caffeine or sugar content). This may indicate that there is some other factor associated with sodas besides caffeine or fructose that is responsible for the observed effects (9, 32). Adding to the complexity, studies in rats have shown that estrogen protects against the development of hyperinsulinemia associated with high fructose intake, explaining why gout is more prevalent in men than in reproductive-aged women (33). Whether it is dietary fructose in sodas or some other component that may affect reproductive hormone levels and ovulatory function in premenopausal women and by what mechanism needs to be elucidated via further research.

Purpose of the Study

The main objectives of this study are: 1) to determine prospectively if caffeine or caffeinated beverages and 2) fructose and fructose-rich beverages are related to serum concentrations of reproductive hormones and incident anovulation in a cohort of 259

healthy, premenopausal women using a standardized method to time serum sample collections. We also wished to assess the validity and reproducibility of the Food Frequency Questionnaire for measuring caffeine and caffeinated beverage compared to repeated 24-hour dietary recalls.

The overall goals for this study are to both improve the methodology in assessing the effect of caffeine and caffeine related beverage intake on health outcomes for premenopausal women and to add to the body of research that seeks to concur on a safe threshold of caffeine and caffeine related beverage intake for women of premenopausal age.

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CHAPTER 2

CAFFEINATED BEVERAGE INTAKE AND REPRODUCTIVE HORMONES AMONG PREMENOPAUSAL WOMEN IN THE BIOCYCLE STUDY

Abstract

Caffeinated beverages are widely consumed among women of reproductive age but their association with reproductive hormones, and whether race modifies any such associations, is not well understood. We assessed the relationship between caffeine and caffeinated beverage intake and reproductive hormones in healthy premenopausal women, evaluating potential effect modification by race. Participants (*n*=259) were followed for up to 2 menstrual cycles, providing fasting blood specimens for hormonal assessment at up to 8 visits per cycle and 4 24-hour dietary recalls per cycle. Weighted linear mixed models and nonlinear mixed models with harmonic terms were used to estimate associations between caffeine and hormone concentrations, adjusting for age, adiposity, physical activity, energy and alcohol intake, and perceived stress. Based on *a priori* assumptions, an interaction between race and caffeine was tested, and stratified results are presented. Caffeine intake ≥200 mg/day was inversely associated with free estradiol concentrations among white women (β=-0.15 [95% confidence interval (CI): -0.26, -0.05] and positively associated among Asian women (β=0.61 [95%

CI: 0.31, 0.92]. Caffeinated soda and green tea intake ≥1 cup/day was positively associated with free estradiol concentrations (β=0.14 [95% CI: 0.06, 0.22] and β=0.26 [95% CI: 0.07, 0.45]) among all races. Moderate consumption of caffeine was associated with reduced estradiol concentrations among white women, while caffeinated soda and green tea intake were associated with increased estradiol concentrations among all races. Further research on the association between caffeine and caffeinated beverages and reproductive hormones, and whether these relationships differ by race, is warranted.

Introduction

Caffeine intake by women of childbearing age is common in the US. Approximately 89% of women aged 18-34 consume on average 166 mg/day of caffeine (equivalent to 1.5 to 2 cups of caffeinated coffee) from a variety of sources but mostly from caffeinated beverages (1, 2). Despite the prevalence of intake, research relating caffeine and reproductive hormone concentrations among premenopausal women is limited and inconclusive (3-6). Inconsistent results may partially be due to the fact that endocrine dynamics of female reproductive hormones (7, 8) and caffeine metabolism (9, 10) are known to have interethnic variability. The association between caffeine and hormones is of interest, as persistent elevation or insufficiency of reproductive hormones during the premenopausal years may not only contribute in the long term to the etiology of certain diseases such as breast, endometrial, and ovarian cancer (11-13) but may also in the short term affect ovulatory function (15)(16). Further understanding of these associations can inform the development of appropriate guidelines regarding consumption levels for women of reproductive age (17).

A variety of pathophysiological effects of caffeine and components of caffeinated beverages on sex hormones and ovulatory function exist. Animal models suggest that caffeine can inhibit oocyte maturation or enhance steroid production via inhibition of phosphodiesterase (4, 18) or alternatively, may interfere with estrogen metabolism via inhibition of aromatase, the key enzyme responsible for converting androgens to estrogen (6, 19). Studies in women have suggested that caffeine may have a positive (4), inverse (6), or null association with estradiol (E2) (5) but has no effect on ovulatory function (16, 20, 21), although no studies to date have prospectively measured caffeine intake at multiple time points and directly measured ovulation. Both caffeine and E2 are metabolized by the hepatic enzyme P450 1A2 (CYP1A2) (22, 23). Polymorphisms of CYP1A2 have been linked to variability in caffeine clearance (24) and serum E2 concentrations (25), and have been shown to modify relationships between caffeine intake and adverse health outcomes (26, 27). Estrogen and caffeine metabolism and risk of breast and ovarian cancer have also been shown to differ between whites and Asians (28-30). It is unknown whether differences in caffeine consumption and metabolism could partially explain these differences.

The primary objective of this study is to determine whether caffeine and its associated beverages (coffee, tea, and soda) are related to serum concentrations of reproductive hormones in a cohort of 259 healthy, premenopausal women, and whether these associations differ by race. Our secondary objective is to determine whether caffeine and its associated beverages are associated with incident anovulation.

Subjects and Methods

Study Population

The BioCycle Study, conducted in 2005-2007, followed women from western New York State for 1 (*n*=9) or 2 (*n*=250) complete menstrual cycles. The study population, materials and methods have been previously described in detail (31). In summary, healthy women aged 18-44 had to be regularly menstruating (self-reported cycle length between 21 and 35 days for each menstrual cycle in the past 6 months) and not currently using hormonal contraception (and for the 3 months prior to study entry) to participate. Of 449 women who were screened, 318 met the eligibility criteria, of whom 276 enrolled. Seventeen women (6%) withdrew before completing the study (31). Women reporting conditions known to affect menstrual cycle function such as polycystic ovary syndrome, uterine fibroids, or known ovulatory disorders were excluded. The University at Buffalo Health Sciences Institutional Review Board (IRB) approved the study and served as the IRB designated by the National Institutes of Health for this study under a reliance agreement. A written informed consent was obtained from all participants.

Hormone Assessment

Women provided fasting blood specimens on up to 8 visits per cycle, with visit timing assisted by use of fertility monitors to correspond to menstruation, mid-follicular, late follicular, luteinizing hormone (LH)/follicle-stimulating hormone (FSH) surge, ovulation, early luteal, mid-luteal, and late luteal phases (32). Total E2 was measured via radio immunoassay while progesterone, LH, FSH, sex hormone binding globulin (SHBG), and insulin were measured using solid-phase competitive chemiluminescent

enzyme immunoassay (Immulite 2000). Albumin assay was tested on the Beckman LX20 auto analyzer using bromcresol purple methodology. Calculation of free E2 (i.e., bioavailable E2) was performed via the equation proposed by Sodergard et al. using total E2, SHBG and albumin concentrations (33). All hormonal analyses were conducted by Kaleida Health Laboratories in Buffalo, New York. Across the study period, the coefficient of variation (CV) for these tests was <10% for E2, SHBG, and insulin, <5% for LH, FSH, and albumin, and <14% for progesterone. Insulin resistance was calculated using the homeostasis model assessment (34). Total cholesterol was measured in serum at each clinic visit using an LX20 automated chemistry analyzer (Beckman, Brea, CA), with a CV of <5%. We defined anovulation as any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum LH peak on the mid- or late-luteal phase visits (*n*=42 of 509 cycles (8.3%)). Study protocol compliance was high, with 94% of the participants completing 7 or 8 visits per cycle.

Dietary Assessment

Participants completed a 24-hour dietary recall (24HDR) at the clinic during the 4 visits corresponding to menstruation, mid-follicular phase, ovulation, and mid-luteal phase. Food and beverage intake was collected and nutrient data were analyzed using the Nutrition Data System for Research (NDSR, 2005, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN). The NDSR program computed the nutrients (i.e., total energy) and non-nutrients (i.e., caffeine) along with beverage components (i.e., coffee) consumed for each day of intake. Further abstraction was done from the raw 24HDR data to discriminate between caffeinated, decaffeinated, or noncaffeinated varieties of coffee, tea (black or green), and soda since this information is not included in

standard NDSR output. Eighty-seven percent of the participants completed 4 dietary recalls per cycle; 99% completed 3.

Covariate Assessment

At study enrollment, waist-to-hip ratio was obtained using standardized protocols by trained study staff, while age, self-identified race, smoking, alcohol intake, reproductive history, and perceived stress were obtained using validated questionnaires (31). For prospectively measured covariates, participants were provided a diary where they were instructed to record daily vigorous exercise (minutes), cigarettes smoked (number), sexual intercourse (yes/no), sleep (hours and minutes), and medication intake (type, dose, units, and number of times per day). Caffeine from medications (primarily from over-the-counter preparations with nonsteroidal anti-inflammatory drugs) was averaged for each phase over the 2 cycles and added to the caffeine calculated from the 8 24HDRs. Eighty-nine percent of participants completed at least 75% of their daily diaries.

Statistical Analyses

Descriptive characteristics of the study population were compared between tertiles of daily caffeine intake, averaged over the 2 cycles under study, and anovulatory status. We assessed differences using analysis of variance per the Satterthwaite method for unequal variances and exact chi-square tests where appropriate (35). Reproductive hormones and serum cholesterol concentrations were log transformed for statistical analyses. Percent of women consuming caffeinated and/or decaffeinated/noncaffeinated coffee, tea, and soda were also generated, based on average intake across the 2 cycles, in

addition to caffeine source, based on total caffeinated food, beverage, or medication items reported.

Weighted linear mixed models were used to evaluate the association between visit-specific caffeine, coffee, tea, or soda intake and log serum concentrations of free and total E2, luteal progesterone, LH, and FSH. Generalized linear mixed models were used to estimate the odds of anovulation based on caffeine and caffeinated beverage consumption. These random-intercept models were chosen to account for between-women variation in baseline hormone concentrations and within-woman correlations of cycles. Recommended limits of caffeine for reproductive-aged women, ≥200mg/day versus <200mg/day (including no exposure) (36); and relevant cut-points for coffee, tea, or soda intake, ≥ 1 cup/day versus < 1cup/day (including no exposure) (4) were assessed. Models were restricted to ovulatory cycles (n=467), as the hormonal patterns for anovulatory cycles are distinctly different from ovulatory cycles.

Based on previous evidence for potential biologic effect modification (4, 37), we tested for interactions between caffeine or beverage exposure and both race and dietary cholesterol intake using the likelihood ratio test (α=0.10). Lucero et al. reported that women whose cholesterol consumption was > 217 mg/day had higher E2 concentrations compared with women whose cholesterol intake was ≤ 217 mg/day (4). Thus, we tested whether there was an interaction between caffeine and cholesterol intake at this cut point (near our participants' average intake of 214 mg/day). In addition to race and dietary cholesterol, we also separately evaluated effect modification by age and waist-to-hip ratio. Stratified analyses (using models with relevant interaction terms) are presented where significant effect modification was found. Potential confounders were determined

a priori using directed acyclic graphs (DAGs) (38). Age, race, waist-to-hip ratio, daily exercise, perceived stress, total energy and alcohol intake (all continuous) were included in our final models. Additional covariates including other caffeinated beverage intake (i.e., adjusting for caffeinated soda and tea when investigating coffee), cigarette use, reproductive history (i.e., gravidity and past oral contraceptive use), sleep, sexual activity, dietary intake (i.e., fiber; cholesterol; and percent calories from carbohydrate, fat and protein), serum cholesterol, and insulin resistance were considered but did not appreciably alter the estimates (39). Based on our proposed DAG, the minimum set of confounders we adjusted for takes into account all sources of measured and known confounding. Since E2 concentrations change over the cycle in response to complex feedback mechanisms with other hormones, traditional regression adjustment for LH, FSH, and progesterone is inadequate. Therefore, we present models that additionally adjust for other reproductive hormones (e.g., progesterone, FSH, LH) through stabilized inverse probability of exposure weights (40, 41).

To assess how caffeine and caffeinated beverage intake affect hormonal patterns, we applied nonlinear mixed models with harmonic terms. While the linear mixed models allow for estimation of mean differences, these harmonic models can additionally evaluate differences in amplitude (i.e., difference between nadir and peak concentrations) and timing of phase shifts while taking into account between and/or within subject variation (42).

Sensitivity analyses were conducted to assess the effects of continuous and varying cut points of caffeine (tertiles and 100 mg increments) or caffeinated beverage (1 cup increments) intakes on reproductive hormones and anovulation. For reproductive

hormones, we assessed effects of including anovulatory cycles or excluding smokers. For anovulation, we assessed the effects of caffeine and caffeinated beverages on the less conservatively defined anovulation (i.e., cycles with progesterone concentrations ≤ 5 ng/mL) or when excluding smokers. Analyses were performed in SAS version 9.2 (SAS Institute, Cary, NC).

Results

Caffeine and Beverage Consumption

Mean caffeine intake across both cycles was 90.9 mg/day (range: 0.0 to 475.4 mg/day). Caffeine intake was positively associated with age; white race; cigarette use; energy, alcohol, and fiber intake; serum FSH; and serum cholesterol concentrations and inversely associated with nulligravidity, perceived stress, and insulin resistance. Anovulation was inversely associated with age, sleep, alcohol, and caffeine intake, total and free E2, and luteal progesterone and positively associated with nulligravidity (Table 2.1).

Over 2 cycles, 49% consumed coffee (88% exclusively caffeinated, 1% exclusively decaffeinated, and 11% caffeinated and decaffeinated), 60% consumed tea (87% exclusively caffeinated, 3% exclusively decaffeinated, and 10% caffeinated and decaffeinated), and 70% consumed soda (18% exclusively caffeinated, 20% exclusively noncaffeinated, and 62% caffeinated and noncaffeinated) (Figure 2.1). Few women consumed energy drinks (2%), so caffeinated energy drink consumption was combined with caffeinated soda consumption. Overall, 66% of caffeine intake came from coffee,

Table 2.1: Characteristics of women participating in the BioCycle Study by average caffeine intake and anovulatory status[1]

		Caffeine Intake (mg/day)				Anovulatory[2]		
	Total	**<25**	**25 – 105**	**> 105**	***P***	**Yes**	**No**	***P***
Number of participants [*n* (%)]	259	86 (33.2)	84 (32.4)	89 (34.4)		35 (13.5)	224 (86.5)	
Caffeine (mg/day)	90.9 ± 94.0	10.9 ± 8.1	58.0 ± 23.7	199.4 ± 79.7	<0.001	60.2 ± 80.0	95.7 ± 95.8	0.04
Demographic/Lifestyle								
Age (years)	27.3 ± 8.2[3]	23.4 ± 5.8	26.5 ± 8.0	31.7 ± 8.3	<0.001	22.5 ± 5.6	28.0 ± 8.3	<0.001
Race[*n* (%)]					<0.001			0.27
White	154 (59.5)	37 (43.0)	49 (58.3)	68 (76.4)		20 (57.1)	134(59.8)	
Black	51 (19.7)	31 (36.1)	14 (16.7)	6 (6.7)		8 (22.9)	43 (19.2)	
Asian	37 (14.3)	15 (17.4)	13 (15.5)	9 (10.1)		7 (20.0)	30 (13.4)	
Other	17 (6.6)	3 (3.5)	8 (9.5)	6 (6.7)		0 (0.0)	17 (7.6)	
BMI (kg/m^2)	24.1 ± 3.9	23.4 ± 3.6	24.6 ± 4.1	24.2 ± 3.8	0.11	23.4 ± 3.8	24.2 ± 3.9	0.25
Waist-to-hip ratio	0.75 ± 0.06	0.75 ± 0.06	0.75 ± 0.05	0.75 ± 0.05	0.90	0.75 ± 0.05	0.75 ± 0.06	0.51
Nulligravid [*n* (%)]	177 (69.4)	71 (83.5)	62 (74.7)	44 (50.6)	<0.001	28 (84.9)	149 (67.1)	0.04
Perceived Stress Score	20.2 ± 6.8	21.7 ± 74	20.3 ± 6.1	18.6 ± 6.5	0.01	20.2 ± 6.9	20.2 ± 6.8	0.99
Cigarette Use [*n* (%)]					0.02			0.79
No	218 (84.2)	80 (93.0)	67 (79.8)	71 (79.8)		30 (85.7)	188 (83.9)	
Yes	41 (15.8)	6 (7.0)	17 (20.2)	18 (20.2)		5 (14.3)	36 (16.1)	
Sleep (hours)	7.2 ± 0.8	7.1 ± 0.9	7.2 ± 0.7	7.2 ± 0.7	0.80	6.8 ± 0.7	7.2 ± 0.8	0.01
Daily exercise (min/day)	14.7 ± 21.9	10.9 ± 12.7	16.7 ± 27.0	16.5 ± 23.4	0.14	20.4 ± 25.2	13.9 ± 21.3	0.10
Sex (intercourse/day)	0.09 ± 0.13	0.07 ± 0.12	0.08 ± 0.12	0.11 ± 0.14	0.10	0.05 ± 0.11	0.09 ± 0.13	0.09
Diet								
Total energy (kcal)	1613.3 ± 367.3	1519.6 ± 342.2	1600.0± 351.5	1716.5 ± 382.6	0.002	1621.2 ± 424.7	1612.1 ± 358.6	0.89
Alcohol (grams)	2.8 ± 5.5	1.0 ± 1.9	2.3 ± 4.7	4.9 ± 7.4	<0.001	1.0 ± 2.1	3.0 ± 5.8	<0.001

Table 2.1 continued

		Caffeine Intake (mg/day)				Anovulatory[2]		
	Total	**<25**	**25 – 105**	**> 105**	***P***	**Yes**	**No**	***P***
Carbohydrates (% calories)	50.8 ± 7.3	51.7 ± 7.5	51.3 ± 7.3	49.4 ± 6.9	0.09	49.9 ± 8.4	50.9 ± 7.1	0.46
Protein (% calories)	15.8 ± 3.1	16.0 ± 3.7	15.8 ± 3.0	15.7 ± 2.6	0.86	16.3 ± 3.8	15.7 ± 3.0	0.30
Fat (% calories)	33.8 ± 5.6	33.3 ± 5.5	33.5 ± 5.7	34.6 ± 5.5	0.25	35.2 ± 5.9	33.6 ± 5.5	0.12
Cholesterol (mg/day)	213.3 ± 106.7	196.8 ± 112.0	220.9 ± 111.6	222.1 ± 95.4	0.21	231.3 ± 128.1	210.5 ± 103.3	0.28
Fiber (g/day)	13.6 ± 5.6	12.7 ± 5.5	13.2 ± 6.1	15.0 ± 4.8	0.02	15.7 ± 8.2	13.3 ± 5.0	0.11
Reproductive Hormones								
Total E2 (ρg/mL)	104.3 (83.3–134.4)[4]	111.1 (83.3–144.8)	102.7 (83.9–131.6)	101.6 (84.5–128.8)	0.57	60.3 ± 1.4	90.0 ± 1.4	<0.001
Free E2 (ρg/mL)	1.3 (1.1–1.6)	1.3 (1.1–1.7)	1.3 (1.1–1.5)	1.3 (1.0–1.5)	0.24	1.0 ± 0.3	1.4 ± 0.5	<0.001
Luteal Progesterone (ng/mL)	3.4 (2.5–4.4)	3.3 (2.5–4.5)	3.0 (2.3–4.2)	3.7 (2.8–4.4)	0.19	0.69 ± 1.7	1.5 ± 1.5	<0.001
LH (ng/mL)	9.2 (7.5–11.4)	9.2 (7.7–11.4)	9.1 (7.5–11.4)	9.2 (7.5–11.3)	0.59	6.7 ± 15	6.0 ± 1.4	0.27
FSH (mIU/mL)	6.0 (5.1–7.0)	5.7 (4.6–6.6)	6.5 (5.2–7.1)	7.0 (5.4–7.5)	<0.001	5.3 ± 1.3	5.4 ± 1.3	0.78
Other biomarkers								
Cholesterol (mg/dL)	163.2 (147.4–177.9)	159.8 (145.3–176.9)	158.9 (146.1–176.4)	169.1 (153.6–186.3)	0.04	161.8 ± 25.3	164.8 ± 27.4	0.54
Insulin resistance (mmol/L)	1.5 (1.1–2.1)	1.7 (1.2–2.6)	1.5 (1.2–2.1)	1.3 (1.0–1.9)	0.02	2.0 ± 1.1	1.8 ± 1.3	0.42

E2, estradiol; LH, luteinizing hormone; FSH, follicle-stimulating hormone

[1] Analysis of variance for continuous variables and exact chi-square tests for categorical variables were used to test associations between caffeine intake or ovulation status. Reproductive hormones and serum cholesterol were log transformed for normality for statistical analyses.
[2] Defined as having at least 1 anovulatory cycle over the 2-cycle study period.
[3]Mean ± *SD*; all such values.
[4]Median (Interquartile Range); all such values.

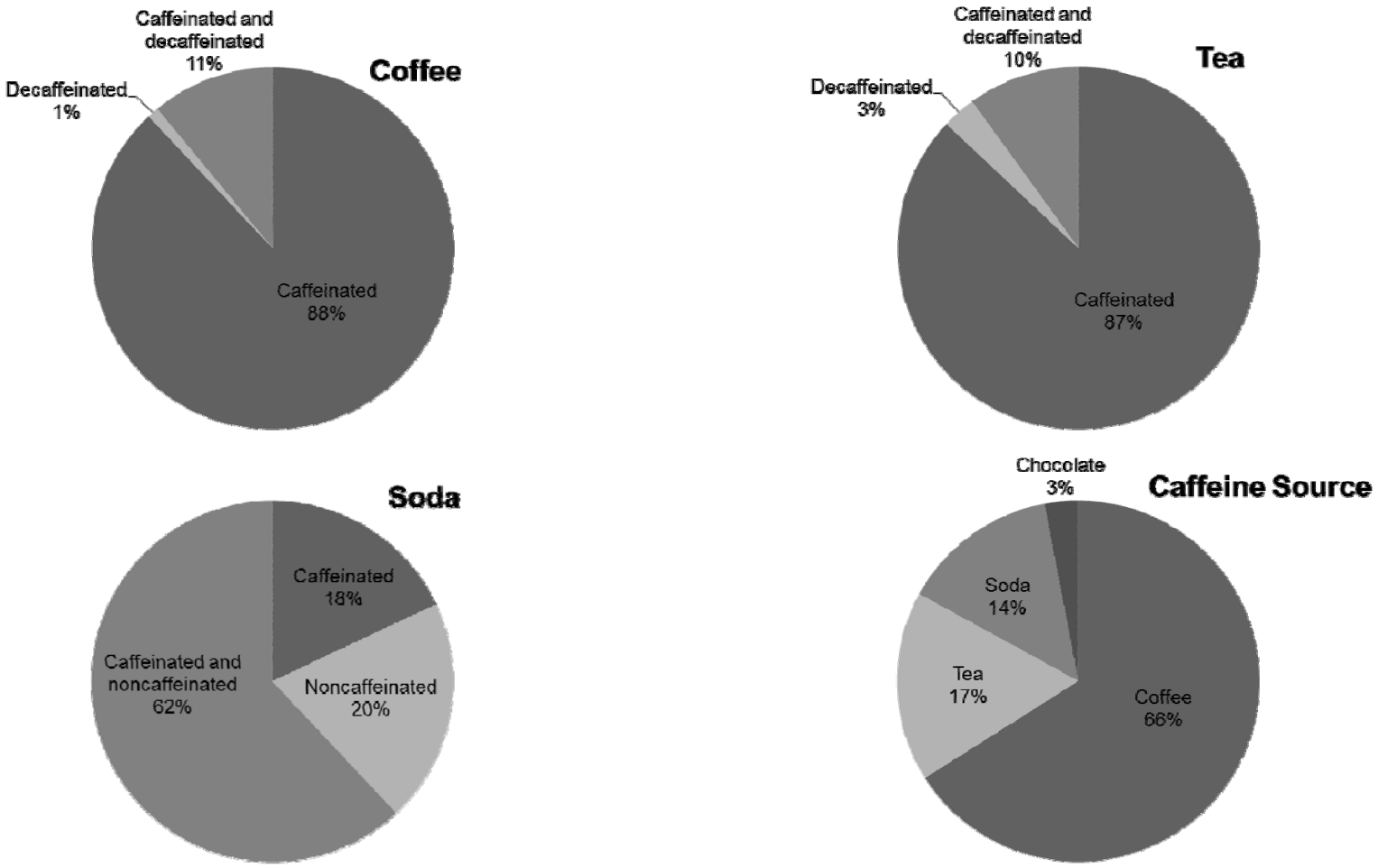

Figure 2.1 Percent of women consuming caffeinated and/or decaffeinated/noncaffeinated beverages, based on average intake captured by 8 24-hour dietary recalls across two menstrual cycles (*n*=259 women); and caffeine source, based on total caffeinated food, beverage, or medication items reported (*n*=3079 items)

17% from tea, 14% from soda, 3% from chocolate, and 0.003% from caffeinated medications (Figure 2.1).

Fifty-eight percent of whites reported consuming coffee, followed by Asians (46%) and blacks (25%). While roughly half of all races reported consuming black tea, 27% of Asians reported consuming green tea, followed by whites (18%) and blacks (12%). Blacks reported the highest frequency of soda consumption (75%), followed by whites (71%) and Asians (59%). Among whites, 70% caffeine came from coffee, 14% from tea, 13% from soda, 3% from chocolate, and 0.004% from caffeinated medications; while among blacks, 33% caffeine came from coffee, 40% from tea, 23% from soda, 4% from chocolate, and 0.001%; and among Asians, 60% caffeine came from coffee, 25% from tea, 12% from soda, and 4% from chocolate (no caffeinated medication was reported among Asians).

Reproductive Hormones

Interactions between race and caffeine (≥200mg/day versus <200mg/day) intake on total and free E2, and LH concentrations were significant (likelihood ratio test, P=0.01, P=0.02, and P=0.01, respectively). Similar effect modification was seen between race and coffee (≥1 cup/day versus <1 cup/day) intake on total and free E2, LH, and FSH (P=0.06, P=0.14, P=0.01, and P=0.03). No significant interactions were found between caffeine and cholesterol intake, age, or waist-to-hip ratio on reproductive hormone concentrations.

We observed that white women who consumed on average ≥200 mg/day of caffeine had lower free (and total) E2 concentrations (free E2: β=-0.15 [95% CI: -0.26, -0.05]) compared to those consuming <200 mg/day, after adjusting for age, waist-to-hip

ratio, perceived stress, daily exercise, energy and alcohol intake, and FSH, LH, and progesterone concentrations (Table 2.2**)**. In contrast, black and Asian women who consumed ≥ 200 mg/day of caffeine had elevated free (and total) E2 (free E2 for blacks: β=0.27 [95% CI: -0.01, 0.56]) and (free E2 for Asians: β=0.44 [95% CI: 0.13, 0.74]). Additionally, black women who consumed ≥200 mg/day of caffeine had reduced FSH (β=-0.36 [95% CI: -0.57, -0.14]) while Asian women had elevated LH (β=0.52 [95% CI: 0.19, 0.85]). Sensitivity analyses showed some evidence of a dose-response: white women who consumed ≥400 mg/day of caffeine (n=17) had lower free (and total) E2 concentrations (free E2, β=-0.39 [95% CI: -0.82, 0.04]) compared to white women who consumed less (data not shown). Too few blacks and Asians reported higher levels of caffeine intake to adequately assess dose-response.

While no statistically significant associations between coffee intake ≥ 1 cup/day and reproductive hormones for whites and blacks were found, Asian women who consumed ≥ 1 cup coffee per day had elevated free (and total) E2 concentrations (free E2, β=0.26 [95% CI: 0.07, 0.44]) (Table 2.2). Assessment of continuous coffee intake (cups/day) showed similar results. As shown in Table 2.3, green (but not black) tea intake ≥ 1 cup/day was associated with elevated free (and total) E2 (free E2, β=0.26 [95% CI: 0.07, 0.45]) after adjusting for age, waist-to-hip ratio, perceived stress, daily exercise, energy and alcohol intake, and FSH, LH, and progesterone concentrations. Additionally, caffeinated soda intake ≥ 1 cup/day was associated with elevated free (and total) E2: (free E2, β=0.14 [95% CI: 0.06, 0.22]) and LH (β=0.13 [95% CI: 0.04, 0.21]) (Table 2.3).For each 1 cup increase in green tea consumption, free (and total) E2 concentrations increased (free E2, β=0.09 [95% CI: 0.02, 0.16]) (data not shown). Sensitivity analyses

Table 2.2: Mean difference in log serum concentrations of reproductive hormones stratified by race of participants according to caffeine and coffee intake (*n*=433 ovulatory cycles)

WHITE (n=277 cycles)	**Caffeine ≥200mg/day vs. <200 mg/day**		**Coffee ≥ 1 cup/day vs. < 1 cup/day**	
Log Hormone	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
E2 (ρg/mL)	-0.09 (-0.19, 0.005)	-0.14 (-0.25, -0.03)	-0.04 (-0.14, 0.05)	-0.06 (-0.16, 0.03)
Free E2 (ρg/mL)	-0.11 (-0.21, -0.01)	-0.15 (-0.26, -0.05)	-0.05 (-0.14, 0.04)	-0.03 (-0.07, 0.01)
Luteal Progesterone (ng/mL)	-0.01 (-0.24, 0.23)	0.00 (-0.27, 0.27)	-0.06 (-0.26, 0.15)	0.04 (-0.17, 0.26)
FSH (mIU/mL)	-0.02 (-0.10, 0.05)	-0.04 (-0.12, 0.04)	0.03 (-0.05, 0.10)	0.02 (-0.05, 0.10)
LH (ng/mL)	-0.04 (-0.15, 0.06)	-0.12, (-0.23, -0.002)	-0.05 (-0.15, 0.05)	-0.06 (-0.16, 0.03)
BLACK (n=92 cycles)	**Caffeine ≥200mg/day vs. <200 mg/day**		**Coffee ≥ 1 cup/day vs. < 1 cup/day**	
Log Hormone	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
E2 (ρg/mL)	0.14 (-0.16, 0.45)	0.24 (-0.05, 0.53)	-0.03 (-0.28, 0.22)	-0.03 (-0.25, 0.20)
Free E2 (ρg/mL)	0.17 (-0.14, 0.47)	0.27 (-0.01, 0.56)	0.003 (-0.24, 0.25)	-0.01 (-0.23, 0.21)
Luteal Progesterone (ng/mL)	0.04 (-0.56, 0.65)	-0.10 (-0.74, 0.55)	-0.05 (-0.56, 0.47)	-0.05 (-0.56, 0.46)
FSH (mIU/mL)	-0.36 (-0.59, -0.14)	-0.36 (-0.57, -0.14)	-0.06 (-0.24, 0.13)	-0.08 (-0.25, 0.09)
LH (ng/mL)	-0.06 (-0.39, 0.28)	-0.08 (-0.39, 0.24)	0.08 (-0.19, 0.35)	-0.003 (-0.25, 0.24)

Table 2.2 continued

ASIAN (n=64 cycles)	Caffeine ≥200mg/day vs. <200 mg/day		Coffee ≥ 1 cup/day vs. < 1 cup/day	
Log Hormone	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
E2 (ρg/mL)	0.44 (0.15, 0.73)	0.61 (0.31, 0.92)	0.35 (0.16, 0.54)	0.32 (0.13, 0.51)
Free E2 (ρg/mL)	0.27 (-0.02, 0.56)	0.44 (0.13, 0.74)	0.28 (0.09, 0.47)	0.26 (0.07, 0.44)
Luteal Progesterone (ng/mL)	0.18 (-0.60, 0.96)	0.28 (-0.60, 1.17)	-0.21 (-0.66, 0.25)	-0.11 (-0.56, 0.34)
FSH (mIU/mL)	0.20 (-0.02, 0.42)	0.17 (-0.06, 0.40)	0.19 (0.04, 0.33)	0.11 (-0.04, 0.25)
LH (ng/mL)	0.43 (0.11, 0.74)[5]	0.52 (0.19, 0.85)	0.27 (0.06, 0.47)	0.14 (-0.06, 0.34)

E2, estradiol; LH, luteinizing hormone; FSH, follicle-stimulating hormone

[1]Coffee includes caffeinated (98%) and decaffeinated (2%); Anovulation is any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum LH peak on the mid or late luteal phase visits. There were 433 ovulatory cycles among whites, blacks and Asians. Analyses were performed using linear mixed effects models on the log scale of hormones. Interactions between race and caffeine (≥200mg/day versus <200mg/day) intake on total and free E2, and LH concentrations were significant (likelihood ratio test, *P*=0.01, *P*=0.02, and *P*=0.01, respectively) as well as between race and coffee (≥1 cup/day versus <1 cup/day) intake on total and free E2, LH, and FSH (*P*=0.06, *P*=0.14, *P*=0.01, and *P*=0.03).
[2]Adjusted for age, waist-to-hip ratio, perceived stress, daily exercise, energy and alcohol intake (all continuous).
[3]Adjusted for age, waist-to-hip ratio, perceived stress, daily exercise, energy and alcohol intake, and relevant phase-specific hormone concentrations using inverse probability of exposure weights.

Table 2.3: Mean difference in log serum concentrations of reproductive hormones according to participants' beverage intake (*n*=467 ovulatory cycles)[1]

	Black Tea ≥ 1 cup/day vs. < 1 cup/day	
Log Hormone	Model 1[2]	Model 2[3]
E2[4] (ρg/mL)	-0.02 (-0.09, 0.06)	0.002 (-0.08, 0.08)
Free E2[4] (ρg/mL)	-0.01 (-0.08, 0.07)	0.01 (-0.08, 0.09)
Luteal Progesterone (ng/mL)	-0.08 (-0.26, 0.11)	-0.05 (-0.26, 0.16)
FSH[4] (mIU/mL)	0.04 (-0.02, 0.10)	0.04 (-0.02, 0.10)
LH[4] (ng/mL)	0.05 (-0.04, 0.14)	0.03 (-0.06, 0.12)
	Green Tea ≥ 1 cup/day vs. < 1 cup/day	
Log Hormone	Model 1[2]	Model 2[3]
E2[4] (ρg/mL)	0.15 (-0.004, 0.31)	0.28 (0.09, 0.47)
Free E2[4] (ρg/mL)	0.14 (-0.02, 0.30)	0.26 (0.07, 0.45)
Luteal Progesterone (ng/mL)	0.02 (-0.32, 0.36)	-0.13 (-0.59, 0.33)
FSH[4] (mIU/mL)	-0.10 (-0.22, 0.02)	-0.04 (-0.18, 0.10)
LH[4] (ng/mL)	0.01 (-0.16. 0.18)	0.09 (-0.11. 0.30)
	Caffeinated Soda ≥ 1 cup/day vs. < 1 cup/day	
Log Hormone	Model 1[2]	Model 2[3]
E2[4] (ρg/mL)	0.11 (0.03, 0.19)	0.15 (0.07, 0.23)
Free E2[4] (ρg/mL)	0.10 (0.03, 0.18)	0.14 (0.06, 0.22)
Luteal Progesterone (ng/mL)	-0.04 (-0.22, 0.15)	-0.10 (-0.30, 0.10)
FSH[4] (mIU/mL)	0.01 (-0.05, 0.07)	0.03 (-0.03, 0.09)
LH[4] (ng/mL)	0.08 (-0.003, 0.16)	0.13 (0.04, 0.21)

E2: estradiol; LH: luteinizing hormone; FSH: follicle stimulating hormone

[1] Anovulation is any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum luteinizing hormone peak on the mid or late luteal phase visits. There were 467 ovulatory cycles among all BioCycle Study participants. Analyses were performed using linear mixed effects models on the log scale of hormones.

Table 2.3 continued

[2] Adjusted for age (continuous); race (white, black, Asian, other); waist-to-hip ratio (continuous); perceived stress (continuous); daily exercise (continuous); energy and alcohol intake (continuous).
[3]Adjusted for age, race, waist-to-hip ratio, perceived stress, daily exercise, energy and alcohol intake, and relevant phase-specific hormone concentrations using inverse probability of exposure weights.

showed that LH concentrations were not associated with each 1 cup increase of caffeinated soda intake, but that free (and total) E2 concentrations increased (free E2, β=0.04 [95% CI: 0.001, 0.08]) (data not shown). Results were similar when including the anovulatory cycles (n=42 cycles) or when restricting analyses to nonsmokers (*n*=218 women) for all models.

Similar relationships, with some differences in patterns, were observed using the nonlinear harmonic models. While intake of ≥ 200 mg/day of caffeine and ≥ 1 cup/day of coffee was associated with decreased mean concentrations of free E2 for white women (β=-0.09 [95% CI: -0.18, -0.01]) and (β=-0.10 [95% CI: -0.17, -0.02]), respectively; increased amplitude in free E2 was observed for Asian women who consumed ≥ 200 mg/day of caffeine (β=0.39 [95% CI: 0.01, 0.77]) and ≥ 1 cup/day of coffee (β=0.29 [95% CI: 0.12, 0.47]), compared to women who consumed less (Figure 2.2 and Figure 2.3). White and Asian women also had a significant free E2 phase shift with coffee intake ≥1 cup/day, although in different directions. Whites peaked later (β=0.05 [95% CI: 0.03, 0.08]) while Asians peaked earlier (β=-0.10 [95% CI: -0.16, -0.04])) (Figure 2.3). The relationship between mean caffeinated soda consumption (≥1 cup versus < 1 cup) and free E2 concentrations among all races mirrored the mixed model results (β=0.11, [95% CI: 0.03, 0.19])) (Figure 2.4). Similar trends were seen for free and total E2 and no further statistically significant differences in hormonal patterns for other caffeinated beverages were found in the harmonic models.

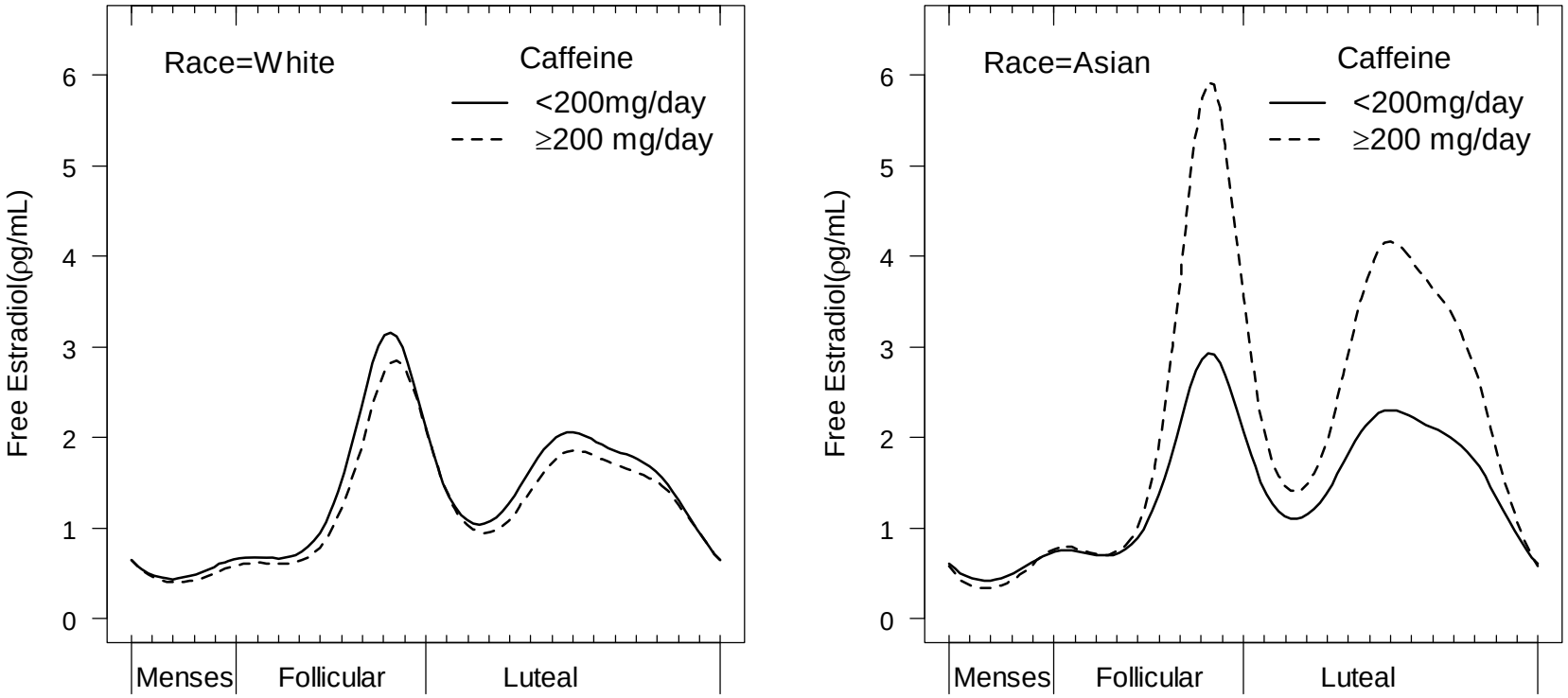

Figure 2.2: Adjusted mean serum concentrations of free estradiol across the menstrual cycle for white and Asian women according to caffeine intake (*n*=277 cycles for whites and *n*=64 cycles for Asians)

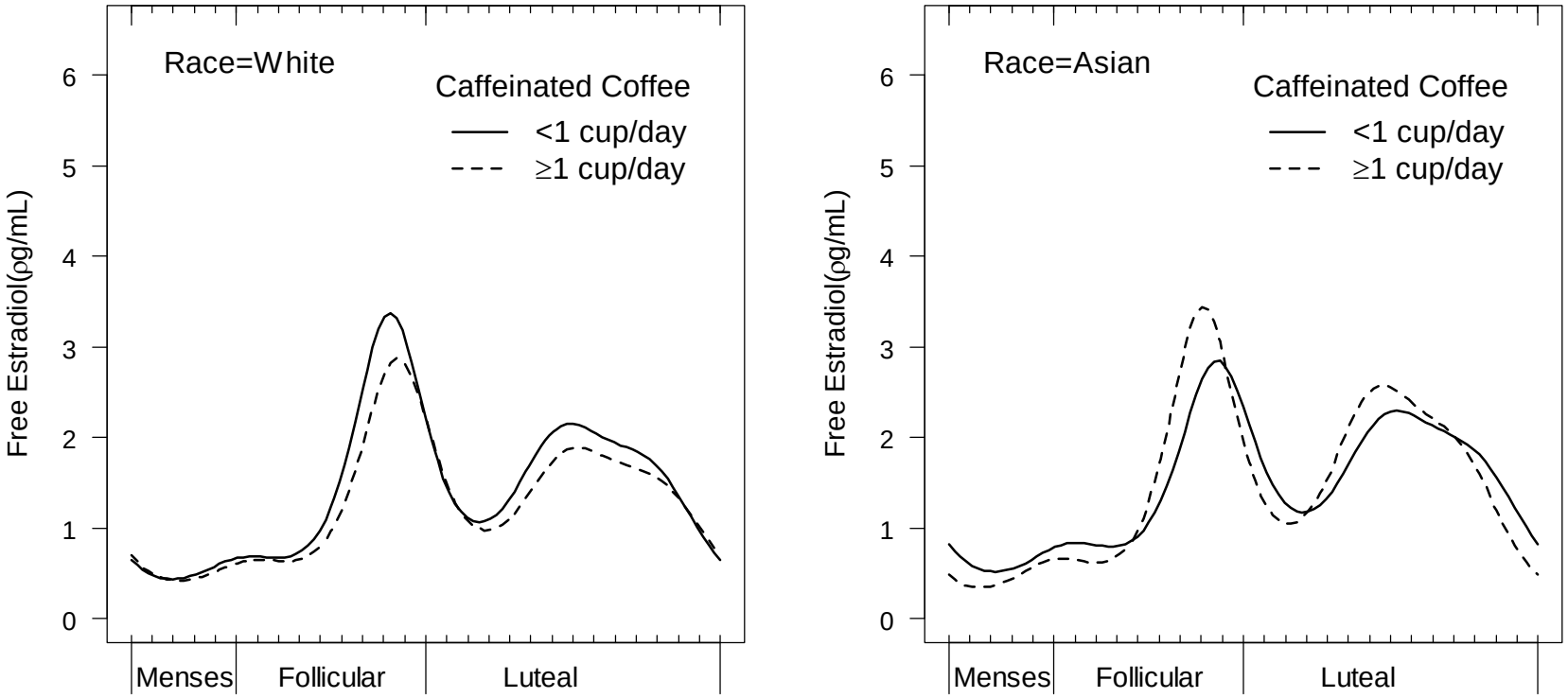

Figure 2.3: Adjusted mean serum concentrations of free estradiol across the menstrual cycle for white and Asian women according to coffee intake (*n*=277 cycles for whites and *n*=64 cycles for Asians)

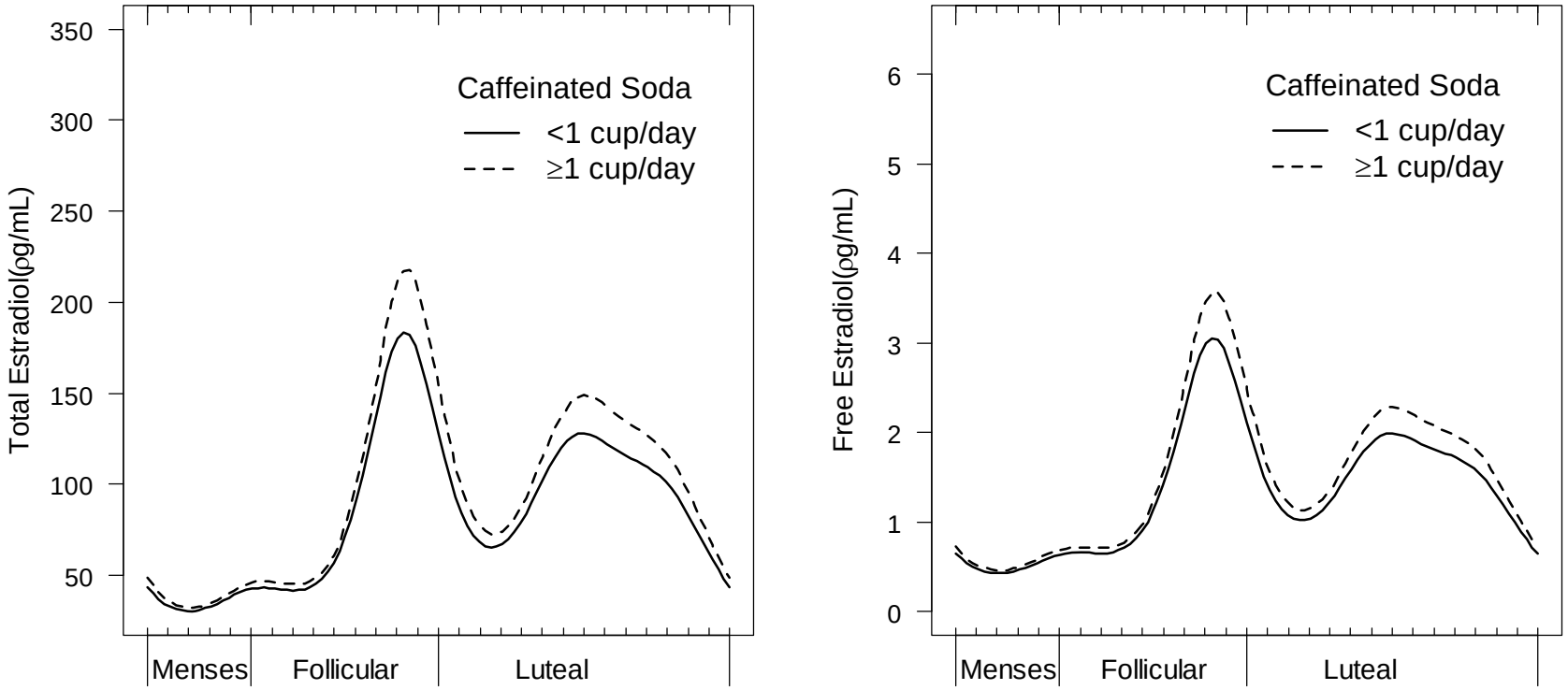

Figure 2.4: Adjusted mean serum concentrations of total and free estradiol across the menstrual cycle of estradiol and free estradiol according to intake of caffeinated soda (*n*=467 cycles)

Anovulation

Green tea intake (≥1 cup/day versus (vs.) <1 cup/day) was associated with increased odds of anovulation (adjusted odds ratio (aOR) = 4.14 [95% CI: 1.26, 13.60]); however, results lacked precision because only 16% (*n*=42 women) reported consuming ≥1 cup/day green tea on at least 1 of their 24HDRs. No further significant associations between caffeine (≥200 mg/day vs. <200 mg/day) and caffeinated beverages (≥1 cup/day vs. <1 cup/day) and ovulatory function were found (Table 2.4). Sensitivity analyses for caffeine (≥100, ≥300, ≥400 mg/day) and all caffeinated beverages (1-cup increments) indicated that 2 or more cups of coffee per day was marginally associated with decreased odds of anovulation (aOR=0.23 [95% CI: 0.05, 1.02]) (data not shown). Assessing anovulation based on the less conservative definition (progesterone ≤ 5 ng/mL) did not alter the findings from our initial analyses nor did restricting the analyses to nonsmokers.

Discussion

We observed that caffeine intake was significantly associated with premenopausal reproductive hormone concentrations and varied across race/ethnicity groups. Higher caffeine intake was associated with decreased free E2 concentrations among whites and increased free E2 concentrations among Asians. Though we observed differences by race, these results are based on a relatively small sample size and should be interpreted with caution. In addition, caffeinated soda and green tea intake was positively associated with increases in total and free E2 concentrations among all races. Caffeine intake above the recommended levels was not associated with anovulation; however, green tea intake ≥ 1 cup/day was associated with increased odds for anovulation.

Table 2.4: Odds of anovulation[1] according to recommended levels of caffeine [≥200mg/day (high) vs <200mg/day (low)] and beverage intake [(≥1 cup/day (high) vs <1 cup/day(low)][2]

		Multivariate adjusted OR (95% CI)[3]
Caffeine	**≥200mg/day vs. <200 mg/day**	0.82 (0.31, 2.21)
Coffee	**≥ 1 cup/day vs. < 1 cup/day**	0.56 (0.22, 1.40)
Black Tea	**≥ 1 cup/day vs. < 1 cup/day**	0.74 (0.32, 1.70)
Green Tea	**≥ 1 cup/day vs. < 1 cup/day**	4.14 (1.26, 13.60)
Caffeinated Soda	**≥ 1 cup/day vs. < 1 cup/day**	0.76 (0.30, 1.94)

[1]Defined as peak progesterone concentrations ≤5 ng/mL and no observed serum LH peak on days 22 or 27 of standardized 28-day cycle (*n*=42 of 509 cycles (8.3%)). Twenty-eight women had 1 anovulatory cycle; 7 women had 2 anovulatory cycles.
[2] Intake assessed at 4 times each cycle: menses, mid-follicular, ovulation and mid-luteal clinic visits via 24-hour dietary recall. Analyses performed using generalized linear mixed models.
[3]Adjusted for age (continuous); race (white, black, Asian, other); waist-to-hip ratio (continuous); perceived stress (continuous); daily exercise (continuous); energy and alcohol intake (continuous).

Our finding of an inverse association between caffeine and free E2 concentrations in premenopausal white women concurs with 2 studies. Kotsopoulos et al. examined free E2 plasma concentrations among 524 predominately white (13) women and found an inverse association between caffeine and luteal free E2 (6). Similarly, Choi et al. found a significant inverse association between increased caffeine intake and decreased peak E2 in 2474 women (race not specified) undergoing infertility treatment (43). In contrast, Lucero et al. found caffeine intake associated with increasing early follicular E2 in 498 predominately white (97%) premenopausal women (4). Kinney et al. found no association between caffeine and early follicular phase total E2 in a study of 188 predominately white (95%) premenopausal women (5), while Nagata et al. found no significant association between caffeine and follicular or luteal E2 among college-aged Asian women (n=50) (3). Comparing these latter 3 studies to ours is limited since they measured caffeine via a single food frequency questionnaire and obtained at most 2 serum samples per cycle without using a validated method to time menstrual cycle phase. Since menstrual cycle phase has been shown to affect caffeine metabolism (44), women's caffeine intake may vary, thus demonstrating the need to capture acute versus habitual patterns when assessing the effect of caffeine on reproductive hormone concentrations.

Similar associations of coffee and caffeine on E2 both in our study and others (6) suggest that caffeine is the component influencing estrogen metabolism. While we did not measure testosterone, evidence has shown higher testosterone concentrations with higher caffeine and coffee intake (6), supporting the hypothesis that caffeine's effect on estradiol is via aromatase inhibition (6, 19). Estrogen metabolism has also been shown to differ between premenopausal Asians and whites (28-30). It has been hypothesized that

gene-environment interactions may partially explain these differences (28-30). *CYP1A2* genotypes have been shown to have interethnic variability, with Asians and Africans appearing to metabolize caffeine more slowly than whites (10, 37). Our results showing higher E2 concentrations among Asians and blacks with higher caffeine intake might be due to *CYP* polymorphisms, but corroboration by other studies is lacking, and we did not directly measure genotype. Additionally, race as a construct represents a complex interplay between many social, lifestyle, environmental, and genetic factors. Further studies are needed to explore differences in the effects of caffeine on reproductive hormones by race.

Despite evidence that elevated or insufficient E2 concentrations can inhibit ovulation (15), we found no association between caffeine, coffee, and anovulation. Our finding corroborates with previous studies (16, 20, 21) and suggests that even though moderate caffeine and coffee intake may alter E2, these alterations are not within a range as to affect ovulatory function. Our results are in line with Choi et al.'s finding that despite lower E2 concentrations in women with moderate-to-high caffeine intake, the number of oocytes retrieved did not differ by caffeine category (43). Recent systematic reviews do not support a positive relationship between caffeine consumption and adverse reproductive outcomes (45, 46).

The effects of tea on reproductive hormones have not been well studied. One study, restricted to Asian women, differentiated exposure by tea type and found green tea to be inversely correlated with follicular estradiol (3). In contrast, women of all races in the BioCycle Study (including Asian women) had statistically elevated free E2 for intake of green tea $\geq$ 1 cup/day compared to those who consumed less, and an increased odds of

anovulation, a finding supported by animal research (47). Increases in E2 in response to green tea intake may also lead to an increase in oxidative stress (48), thus requiring more antioxidants to compensate for the increase in oxidative stress. Given that green tea is high in antioxidants and recent evidence suggests that antioxidants adversely affect ovulatory function (49) further research is justified. Caution is warranted regarding the marginal association we found between anovulation and green tea due to limited intake among study participants and lack of comparative studies.

Our finding that soda intake significantly increases E2 concentrations is novel, but mirrors results from animal studies. Celec et al. found intake of 3 different sweetened cola drinks to be associated with increased E2 concentrations in adult male Wistar rats (*n*=40) (50). The only other human study that we are aware of investigating soda and premenopausal E2 concentrations found no effect after adjusting for age, BMI, and cycle length, but restricted their investigation to cola and had a small sample size (n=50) and limited exposure/outcome assessment (3).

The BioCycle Study has several strengths, including multiple measures of hormone assessment over 2 menstrual cycles (using standardized methods to time cycle phase) and multiple measures of not only caffeine and caffeinated beverage intake, but important dietary and lifestyle factors as well. While self-report of diet is subject to measurement error (51-53), our study used multiple validated 24HDRs to reduce the potential for exposure misclassification.

Nevertheless, the study had several limitations including the low percentage of women who consumed ≥ 200 mg/day of caffeine and ≥ 1 cup/day of caffeinated beverages. US premenopausal women daily consume on average 166 mg of total

caffeine, 19 oz of soda, 6 oz of coffee, and 5 oz of tea (1, 54) whereas the BioCycle Study participants daily consumed on average 91 mg of caffeine, 3 oz of soda, 4 oz of coffee, and 3 oz of tea. Additionally, while the BioCycle Study had greater racial diversity than comparable studies (3-6), our study was limited by different sample sizes among the racial groups, which may have limited our power to detect significant differences in some of our subgroup analyses.

In conclusion, within moderate ranges of consumption, caffeine was associated with reduced E2 concentrations among white women and elevated E2 concentrations among Asian women. Caution regarding effect modification by race in this study is warranted due to limited numbers of Asians with high exposure. Understanding the relationship between caffeine and E2 has substantial implications for women's health, both in regard to reproductive health and hormonally dependent cancers. Higher concentrations of E2 are found in women with endometriosis, a well-known risk factor for infertility (55). Additionally, there is evidence for increased risk of breast cancer with increased E2 concentrations among premenopausal women (56) and possibly for endometrial and ovarian cancers as well (12). Furthermore, bone mineral density is known to have interethnic/race variation and may be influenced by sex hormones (57-59). Given these public health implications, further research investigating whether caffeine or other components in caffeinated beverages play a role in reproductive hormone synthesis is needed, as well as evaluation as to whether these relationships differ by race.

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CHAPTER 3

FRUCTOSE-RICH BEVERAGES: REPRODUCTIVE HORMONES AND OVARIAN FUNCTION IN THE BIOCYCLE STUDY

Abstract

Fructose-rich beverages are widely consumed among women of reproductive age but their association with reproductive function is not well understood. Our objective was to assess the association of added sugars, fructose, and beverage intake with reproductive hormones and sporadic anovulation in healthy premenopausal women. Women (*n*=259) in the BioCycle Study were followed for up to 2 menstrual cycles, providing fasting blood specimens at up to 8 visits/cycle and 4 24-hour dietary recalls/cycle. Participants who consumed more added sugar than an average American woman (≥ 73.2 grams/day) or above the 66th percentile in fructose intake (≥ 41.5 grams/day) had elevated free estradiol (E2), follicular stimulating hormone (FSH) and luteinizing hormone (LH) concentrations compared to women who consumed less after adjusting for age, waist-to-hip ratio, race, dietary factors, physical activity, and relevant hormones. No associations were found between intakes above the American Heart Association's recommended limits for added sugar intake (≥ 40 grams/day) and reproductive hormone levels across the cycle. Women who consumed ≥1 cup/day of sweetened soda had elevated free E2 (β=0.15 [95% CI: 0.06, 0.24]. Neither artificially sweetened soda intake nor fruit juice intake ≥1 cup/day was significantly associated with reproductive hormones. No

associations were found between added sugars, fructose or beverage intake and anovulation. Even at moderate consumption levels, added sugars, total fructose, and sweetened soda were associated with elevated E2 concentrations among premenopausal women. Further research into whether fructose alone or in conjunction with other components in sweetened soda is associated with sex hormones is warranted.

Introduction

Sweetened soda intake, the largest contributor of fructose in the American diet (whether sweetened with sugar or high fructose corn syrup), has markedly increased over the last few decades rising from roughly 2 8-oz servings/week in 1942 to roughly 2 8-oz servings/day in 2000 (1). Women of childbearing age in the United States derive on average over 23% of their daily energy from beverage sources (2). Due to overwhelming experimental and epidemiologic evidence indicating that sweetened beverages are associated with weight gain, metabolic syndrome, and cardiovascular risk factors (3-5), the American Heart Association (AHA) recently expanded its recommendation to limit intake of added sugars by proposing specific values depending on age, gender, and physical activity (6). According to the AHA, an adult woman who is moderately physically active, age 19–30, should limit her consumption of added sugars to between 8 to 12 teaspoons/day (≈32–48 grams(g)/day), equivalent to approximately a 12-oz can of nondiet soda, significantly below the current usual intake for an adult American woman of 18.3 teaspoons/day (73.2 g/day) (6).

While both human and animal studies have shown that diets high in fructose result in dyslipidemia and insulin resistance (7-12), well-known risk-factors for hormone and ovulatory disorders (13), research assessing the effects of sweetened sodas and/or

fructose on premenopausal reproductive hormone levels (14, 15) and ovulatory function (13) is sparse. Studies have found no association between soda and premenopausal reproductive hormones, but inferences have been limited by small sample sizes and/or inadequate methods to evaluate the phase of the menstrual cycle when the hormones were measured. Women participating in the Nurses' Health Study who consumed ≥ 2 8-ounce (oz) cups/day of caffeinated soda were recently shown to have an increased relative risk (RR=1.47, 95% CI: 1.09–1.98) for ovulatory disorder compared to women who consumed < 1 8-oz cup/week, (13), but no significant results with total caffeine nor with caffeinated coffee or tea were found. These findings suggest that relevant components may be specific to soda. The relationship between these components and sex hormones is of interest for assessing not only their effects on female reproductive function (13), but also for better understanding hormone-related chronic diseases (16).

The objective of this study is to determine whether added sugars, fructose, and sweetened or artificially sweetened beverage intake are related to serum concentrations of reproductive hormones and ovulatory function in a cohort of 259 healthy, premenopausal women, using a standardized method to time serum sample collections according to the phase of the menstrual cycle.

Subjects and Methods

Study Population

The BioCycle Study, conducted in 2005–2007, followed 259 women from western New York State for up to 2 complete menstrual cycles. The study population, materials and methods have been previously described in detail (17). In summary, healthy women aged 18–44 had to be regularly menstruating (self-reported cycle length between

21 and 35 days for each menstrual cycle in the past 6 months) to participate. Women reporting conditions known to affect menstrual cycle function such as polycystic ovary syndrome, uterine fibroids, or current or recent use of hormonal contraception (i.e., 3 months prior to study entry) were excluded. Women with previously known ovulatory disorders were excluded, but sporadic anovulation (*n*=35 women, 42 cycles) was observed in the study population (18). The University at Buffalo Health Sciences Institutional Review Board (IRB) approved the study and served as the IRB designated by the National Institutes of Health for this study under a reliance agreement. Written informed consent was obtained from all participants.

Hormone Assessment

Women provided fasting blood specimens on up to 8 visits/cycle for 1 (*n*=9) or 2 (*n*=250) menstrual cycles, with visit timing assisted by use of fertility monitors to correspond to menstruation, mid-follicular, late follicular, luteinizing hormone (LH)/follicle stimulating hormone (FSH) surge, ovulation, early luteal, mid-luteal, and late luteal phases (19). Total estradiol (E2) was measured via radio immunoassay while progesterone, LH, FSH, and sex hormone binding globulin (SHBG) were measured using solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000). The albumin assay was tested on the Beckman LX20 auto analyzer using bromcresol purple methodology. Calculation of free E2 (i.e., bioavailable E2) was performed via the equation proposed by Sodergard et al. using total E2, SHBG, and albumin concentrations (20). All hormonal analyses were conducted by Kaleida Health Laboratories in Buffalo, New York. Across the study period, the coefficient of variation (CV) for these tests was <10% for E2, SHBG, and insulin <5% for LH, FSH, and albumin, and <14% for

progesterone. We defined anovulation as any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum LH peak on the mid or late luteal phase visits (*n*=42 of 509 cycles (8.3%)). Study protocol compliance was high, with 94% of the participants completing 7 or 8 visits/cycle.

Dietary Assessment

Participants completed a 24-hour dietary recall (24HDR) at the clinic after fasting blood specimen collection during the 4 visits corresponding to menstruation, mid-follicular phase, ovulation and mid-luteal phase. Food and beverage intake (including sweetened and artificially sweetened (diet) sodas, and citrus and noncitrus fruit juices) was collected and nutrient data were analyzed using the Nutrition Data System for Research (NDSR, 2005, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN). We assessed citrus and noncitrus juice separately since grapefruit juice has been shown to increase endogenous estrogen levels (21). In addition to total sugars and added sugars, the NDSR provides information on daily free fructose and sucrose intake. Since half of the disaccharide sucrose is fructose (which is split from glucose in the small intestine), we calculated total fructose equal to the intake of free fructose plus half the intake of sucrose (22). Further abstraction was done from the raw 24HDR data to discriminate between cola and noncola soda since this information is not included in standard NDSR output and caramel coloring agents in colas are thought to affect health outcomes (23-25). Compliance was high with 87% completing 4 dietary recalls/cycle and 99% completing 3.

Covariate Assessment

At study enrollment, adiposity measurements, including body mass index (BMI) and waist-to-hip ratio were obtained by trained study staff using standardized protocols, while age, race, and reproductive history were obtained using validated questionnaires (17). For prospectively measured covariates, participants were provided a diary where they were instructed to record daily vigorous exercise (minutes) and cigarettes smoked (number). The majority of participants (89%) completed at least 75% of their daily diaries.

Statistical Analyses

Descriptive statistics were compared between tertiles of daily fructose intake and recommended limits (≤ 1 cup) of sweetened soda intake, averaged over the 2 cycles under study. We assessed differences using analysis of variance per the Satterthwaite method for unequal variances and exact chi-square tests where appropriate (26). Reproductive hormone levels were log transformed for statistical analyses. Weighted linear mixed models were used to evaluate the association between added sugars, fructose, soda (sweetened or artificially sweetened; cola and noncola), and juice intake (15) and log serum concentrations of free and total E2, luteal progesterone, LH and FSH. Generalized linear mixed models were used to estimate the odds of anovulation based on fructose, added sugars and beverage consumption. We assessed the association between intakes above the AHA recommended added sugar intake levels for moderately physically active American women, age 19-30, (≥40.0 g/day versus <40.0 g/day) (27), as well as intakes above the usual added sugar intake for American women (≥73.2 g/day versus <73.2 g/day) (6), with hormone concentrations and anovulation. We additionally

compared cutpoints at the 33rd percentile (≥ 28.4 g/day versus <28.4 g/day), 50th percentile (median) (≥ 33.5 g/day versus <33.5 g/day), and 66th percentile (≥ 41.5 g/day versus <41.5 g/day) for total fructose, and (≥ 1 cup/day versus < 1 cup/day) sweetened or artificially sweetened beverages (15). Random-intercepts were included in the models to account for between-women variation in baseline hormone concentrations and within-woman correlations across cycles. Models evaluating reproductive hormones were restricted to ovulatory cycles (18) as the hormonal patterns for anovulatory cycles are distinctly different from ovulatory cycles.

Potential confounders were determined *a priori* using directed acyclic graphs (DAG). Age (continuous), waist-to-hip ratio (continuous), race (white, black, Asian and other), total energy intake (continuous), physical activity (continuous), and a previously described (28) dietary quality score (alternate Mediterranean Diet Score; continuous) were included in our final models. For all models of soda intake, we also adjusted for total caffeine intake. Additional covariates including other beverages consumed, cigarette use, and gravidity were considered but did not appreciably alter the estimates (29), nor did adjusting for BMI versus waist-to-hip ratio. Based on our proposed DAG, the minimum set of confounders we adjusted for accounts for all sources of measured and known confounding. Since E2, progesterone, LH, and FSH levels change over the cycle in response to complex feedback mechanisms with other hormones, traditional regression adjustment for other hormone levels is inadequate. Therefore, we additionally present models that adjust for other reproductive hormones through stabilized inverse probability of exposure weights (30, 31).

To assess how fructose and fructose-rich beverage intake affect hormonal patterns, we used nonlinear mixed models with harmonic terms. While the linear mixed models allow for estimation of mean differences, these harmonic models can additionally evaluate differences in amplitude (i.e., difference between nadir and peak concentrations) and timing of phase shifts while taking into account between and/or within subject variation (32).

Sensitivity analyses were conducted to assess the effects of continuous beverage intake (1 cup increments) on reproductive hormones and anovulation. For reproductive hormones, we additionally assessed effects of including anovulatory cycles. For anovulation, we assessed the effects of fructose or fructose-rich beverages on a less conservative classification of anovulation defined as cycles with progesterone concentrations ≤ 5 ng/mL (*n*=65 of 509 cycles (12.8%)).

Results

Fructose and Beverage Consumption

Mean intake of added sugars and total fructose across both cycles were 57.2 ± 26.9 g/day and 35.4 ± 13.7 g/day, respectively. Fructose intake over the study period was positively associated with black race, total energy intake, percent calories from carbohydrates, added sugars and fiber intake and inversely associated with percent calories from protein. Sweetened soda intake was positively associated with black race, fructose and added sugar intake and both E2 and free E2 concentrations and inversely associated with daily exercise, alcohol, fiber intake, and percent calories from protein (Table 3.1). Over 2 cycles, 69% consumed soda (52% exclusively sweetened, 27%

Table 3.1: Characteristics of women participating in the BioCycle Study by average fructose[1] and sweetened soda intake across 2 menstrual cycles

		Fructose Intake (g/day)				Sweetened Soda Intake		
	Total	**<28.4**	**28.4-41.5**	**>41.5**	***P***	**<1 cup**	**≥ 1cup**	***P***
Number of participants [*n* (%)]	259	86 (33.2)	88 (34.0)	85 (32.8)		244 (94.2)	15 (5.8)	
Demographic/Lifestyle								
Age (years)	27.3 ± 8.2[2]	26.8 ± 8.1	27.0 ± 8.0	28.1 ± 8.6	0.54	27.3 ± 8.3	26.5 ± 7.1	0.71
Race[*n* (%)]					0.02			0.01
White	154 (59.5)	43 (50.0)	60 (68.2)	51 (60.0)		147 (60.3)	7 (46.7)	
Black	51 (19.7)	16 (18.6)	13 (14.8)	22 (25.9)		43 (17.6)	8 (53.3)	
Asian	37 (14.3)	21 (24.4)	10 (11.4)	6 (7.1)		37 (15.2)	0 (0.0)	
Other	17 (6.6)	6 (7.0)	5 (5.7)	6 (7.1)		17 (7.0)	0 (0.0)	
BMI (kg/m^2)	24.1 ± 3.9	23.8 ± 3.9	24.3 ± 3.8	24.1 ± 3.9	0.74	24.0 ± 3.8	25.3 ± 4.1	0.20
Waist-to-hip ratio	0.75 ± 0.06	0.74 ± 0.05	0.75 ± 0.06	0.75 ± 0.05	0.31	0.75 ± 0.06	0.78 ± 0.06	0.08
Nulligravid [*n* (%)]	177 (69.4)	60 (72.3)	63 (72.4)	54 (63.5)	0.35	165 (68.8)	12 (80.0)	0.56
Cigarette Use [*n* (%)]					0.25			0.48
No	218 (84.2)	71 (83.0)	71 (81.0)	76 (89.4)		204 (83.6)	14 (93.3)	
Yes	41 (15.8)	15 (17.4)	17 (19.3)	9 (10.6)		40 (16.4)	1 (6.7)	
Vigorous exercise (min/day)	14.7 ± 21.9	16.9 ± 24.8	15.6 ± 18.7	11.7 ± 21.9	0.26	15.4 ± 22.4	4.7 ± 5.0	<0.001
Diet								
Total energy (kcal)	1613.3 ± 367.3	1378.9 ± 320.5	1649.4 ± 322.0	1813.1 ± 324.0	<0.001	1602.6 ± 366.2	1786.9 ± 353.1	0.06
Alcohol (grams)	2.8 ± 5.5	3.1 ± 7.0	3.1 ± 5.4	2.1 ± 3.3	0.37	2.9 ± 5.6	1.1 ± 1.7	0.004
Carbohydrates (% calories)	50.8 ± 7.3	47.5 ± 7.8	51.3 ± 7.0	53.6 ± 5.5	<0.001	50.7 ± 7.3	52.0 ± 7.0	0.52
Protein (% calories)	15.8 ± 3.1	17.5 ± 3.4	15.6 ± 2.5	14.3 ± 2.5	<0.001	15.9 ± 3.1	14.0 ± 2.1	0.02
Fat (% calories)	33.8 ± 5.6	34.9 ± 6.2	33.6 ± 5.4	32.9 ± 4.9	0.06	33.7 ± 5.6	34.6 ± 6.1	0.56

Table 3.1 continued

		Fructose Intake (g/day)				Sweetened Soda Intake		
	Total	**<28.4**	**28.4-41.5**	**>41.5**	***P***	**<1 cup**	**≥ 1cup**	***P***
Fiber (g/day)	13.6 ± 5.6	11.8 ± 4.3	15.0 ± 6.0	14.1 ± 5.8	<0.001	13.9 ± 5.6	9.4 ± 3.4	0.001
Fructose (g/day)	17.2 ± 9.2	20.8 ± 5.1	34.5 ± 4.1	51.2 ± 7.9	<0.001	34.2 ± 12.9	55.3 ± 11.8	<0.001
Added sugars (g/day)	57.2 ± 26.9	32.8 ± 12.9	55.9 ± 14.7	83.3 ± 23.2	<0.001	55.0 ± 25.6	93.5 ± 22.0	<0.001
Reproductive Hormones								
Total E2 (ρg/mL)	104.3 (83.3–134.4)[3]	100.8 (78.8-132.0)	106.6 (84.6-146.4)	108.8 (84.6-133.8)	0.62	103.1 (82.7-132.7)	121.0 (110.9-152.8)	0.01
Free E2 (ρg/mL)	1.3 (1.1–1.6)	1.3 (1.1-1.6)	1.3 (1.0-1.5)	1.3 (1.1-1.7)	0.43	1.3 (1.0-1.6)	1.6 (1.3-1.9)	0.01
Luteal Progesterone (ng/mL)	3.4 (2.5–4.4)	3.8 (2.4-4.5)	3.1 (2.4-4.1)	3.3 (2.7-4.5)	0.06	3.4 (2.5-4.4)	3.4 (2.9-4.5)	0.28
LH (ng/mL)	9.2 (7.5–11.4)	10.1 (7.9-11.6)	8.8 (7.4-11.4)	9.0 (7.4-10.9)	0.15	9.2 (7.5-11.3)	10.7 (7.2-12.2)	0.74
FSH (mIU/mL)	6.0 (5.1–7.0)	6.0 (5.2-6.9)	6.1 (5.2-7.2)	5.9 (4.9-6.9)	0.15	6.0 (5.2-7.0)	5.2 (4.6-6.7)	0.18

BMI, body mass index; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

[1] Intake assessed at 4 times each cycle: menses, mid-follicular, ovulation and mid-luteal clinic visits via 24-hour dietary recall. Fructose equal to the intake of free fructose plus half the intake of sucrose

[2]Calculated by using student's t-test for continuous variables (Satterthwaite method when variance unequal) and exact chi-square tests for categorical variables. Reproductive hormones were log transformed for normality for statistical analyses.

[3]Mean ± *SD*; all such values.

[4]Median (Interquartile Range); all such values.

exclusively artificially sweetened, and 21% both sweetened and artificially sweetened), and 81% consumed fruit juice (59% exclusively citrus, 11% exclusively noncitrus fruit juice and 30% both citrus and other fruit juice).

Reproductive Hormones

Women above the 66th percentile in fructose intake (41.5 g/day) had elevated free E2, FSH, and LH concentrations compared to women consuming <41.5 g/day (Table 3.2), but no statistically significant differences were found at the 33rd or 50th percentiles (data not shown). While no statistically significant associations were found between consumption above the AHA recommended added sugar intake levels (≥ 40.0 g/day versus < 40.0 g/day) and reproductive hormone levels across the menstrual cycle, those who consumed more added sugar than American women on average (≥ 73.2 g/day) had elevated free and total (marginal) E2, FSH, and LH compared to women who consumed <73.2 g/day after adjusting for age, waist-to-hip ratio, race, total energy intake, physical activity, dietary quality score, and relevant hormones (Table 3.2). Consumption of ≥ 1 cup/day of sweetened soda was associated with elevated free and total E2 concentrations (free E2: β=0.15 [95% CI: 0.06, 0.24]) compared to women who consumed <1 cup/day after multivariate adjustment, including total caffeine intake (Table 3.3). Results were similar for cola and noncola intake, with consumption ≥ 1 cup/day associated with elevated free and total E2 concentrations. Neither intake of artificially sweetened soda nor fruit juice (≥ 1 cup/day versus <1 cup/day) was significantly associated with reproductive hormone concentrations (Table 3.3). Sensitivity analyses showed that for each 1-cup increment in sweetened soda intake, free (and total) E2 increased (free E2:

Table 3.2: Mean difference in log serum concentrations of reproductive hormones according to added sugar and fructose intake (*n*=467 ovulatory cycles)[1]

	Added Sugars ≥ 40.0 vs < 40.0 g/day AHA Recommended Limit		**Added Sugars ≥ 73.2 vs < 73.2 g/day** Usual Intake for American Women		**Fructose ≥ 41.5 vs < 41.5 g/day** 66th Percentile	
Log Hormone	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
Total E2 (ρg/mL)	0.04 (-0.02, 0.10)	0.04 (-0.02, 0.10)	0.07 (0.01, 0.14)[4]	0.05 (-0.01, 0.11)	0.06 (-0.004, 0.12)	0.04 (-0.02, 0.10)
Free E2 (ρg/mL)	0.03 (-0.03, 0.09)	0.04 (-0.02, 0.10)	0.07 (0.01, 0.14)[4]	0.06 (0.004, 0.13)[4]	0.07 (0.01, 0.13)[4]	0.06 (0.002, 0.12)[4]
Luteal Progesterone (ng/mL)	0.08 (-0.07, 0.23)	0.06 (-0.09, 0.21)	0.06 (-0.11, 0.23)	0.07 (-0.11, 0.25)	0.01 (-0.14, 0.1)	-0.11 (-0.27, 0.05)
FSH (mIU/mL)	0.05 (-0.003, 0.10)	0.02 (-0.02, 0.07)	0.05 (-0.004, 0.09)	0.12 (0.08, 0.17)[4]	0.09 (0.05, 0.14)[4]	0.14 (0.08, 0.20)[4]
LH (ng/mL)	0.03 (-0.04, 0.10)	0.02 (-0.04, 0.09)	0.05 (-0.02, 0.13)	0.17 (0.11, 0.24)[4]	0.09 (0.02, 0.16)[4]	0.11 (0.07, 0.16)[4]

E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

[1]Anovulation is any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum LH peak on the mid or late luteal phase visits (*n*=42 cycles). Nutrient intake assessed at 4 times each cycle: menses, mid-follicular, ovulation and mid-luteal clinic visits via 24-hour dietary recall. Fructose equal to the intake of free fructose plus half the intake of sucrose.
[2]Adjusted for age, waist-to-hip ratio, race, total energy intake, dietary quality score, and physical activity using linear mixed effects models on the log scale of hormones.
[3] Adjusted for age, waist-to-hip ratio, race, total energy intake, dietary quality score, physical activity and relevant phase-specific hormone levels using weighted linear mixed effects models on the log scale of hormones with inverse probability of exposure weights.
[4]*P*<0.05

Table 3.3: Mean difference in log serum concentrations of reproductive hormones according to fructose-rich beverage intake (*n*=467 ovulatory cycles)[1]

	Sweetened Soda ≥ 1 vs < 1 cup/day		Artificially Sweetened Soda ≥ 1 vs < 1 cup/day	
Log Hormone	Model 1[2]	Model 1[2]	Model 1[2]	Model 2[3]
Total E2 (ρg/mL)	0.12 (0.04, 0.21)[4]	0.15 (0.06, 0.24)[4]	0.05 (-0.05, 0.15)	0.09 (-0.02, 0.19)
Free E2 (ρg/mL)	0.12 (0.03, 0.21)[4]	0.15 (0.06, 0.24)[4]	0.03 (-0.06, 0.13)	0.06 (-0.04, 0.17)
Luteal Progesterone (ng/mL)	-0.02 (-0.23, 0.18)	0.02 (-0.22, 0.26)	0.03 (-0.19, 0.24)	-0.02 (-0.26, 0.22)
FSH (mIU/mL)	-0.04 (-0.11, 0.02)	0.004 (-0.06, 0.07)	0.00 (-0.07, 0.07)	0.03 (-0.05, 0.11)
LH (ng/mL)	-0.01 (-0.11, 0.08)	0.07 (-0.03, 0.17)	-0.004 (-0.11, 0.10)	0.05 (-0.06, 0.16)
	Cola Soda ≥ 1 vs < 1 cup/day		**Non-Cola Soda ≥ 1 vs < 1 cup/day**	
Log Hormone	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
Total E2 (ρg/mL)	0.09 (0.01, 0.17)[4]	0.13 (0.05, 0.20)[4]	0.09 (0.01, 0.17)[4]	0.12 (0.04, 0.20)[4]
Free E2 (ρg/mL)	0.09 (0.01, 0.16)[4]	0.12 (0.04, 0.20)[4]	0.08 (0.005, 0.16)[4]	0.12 (0.04, 0.20)[4]
Luteal Progesterone (ng/mL)	-0.03 (-0.22, 0.15)	-0.14 (-0.34, 0.07)	-0.03 (-0.22, 0.16)	-0.14 (-0.35, 0.06)
FSH (mIU/mL)	0.01 (-0.05, 0.07)	0.03 (-0.03, 0.09)	0.01 (-0.05, 0.07)	0.05 (-0.01, 0.11)
LH (ng/mL)	0.04 (-0.04, 0.13)	0.10 (0.02, 0.19)	0.04 (-0.04, 0.13)	0.12 (0.04, 0.20)
	Citrus Fruit Juice ≥ 1 vs < 1 cup/day		**Non-citrus Fruit Juice ≥ 1 vs < 1 cup/day**	
Log Hormone	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
Total E2 (ρg/mL)	0.05 (-0.06, 0.15)	0.04 (-0.04, 0.13)	0.04 (-0.05, 0.12)	0.09 (-0.03, 0.20)
Free E2 (ρg/mL)	0.06 (-0.05, 0.16)	0.04 (-0.04, 0.13)	0.03 (-0.05, 0.12)	0.09 (-0.02, 0.21)
Luteal Progesterone (ng/mL)	-0.19 (-0.46, 0.08)	-0.14 (-0.36, 0.08)	-0.17 (-0.37, 0.03)	-0.09 (-0.42, 0.25)
FSH (mIU/mL)	0.03 (-0.05, 0.10)	0.05 (-0.01, 0.12)	0.03 (-0.04, 0.09)	0.07 (-0.02, 0.15)
LH (ng/mL)	0.004 (-0.09, 0.09)	0.06 (-0.04, 0.15)	-0.04 (-0.15, 0.08)	0.05 (-0.08, 0.18)

E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

[1]Anovulation is any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum LH peak on the mid or late luteal phase visits (n=42 cycles). Beverage intake assessed at 4 times each cycle: menses, mid-follicular, ovulation and mid-luteal clinic visits via 24-hour dietary recall.

Table 3.3 continued

[2] Adjusted for age, waist-to-hip ratio, race, total energy intake, dietary quality score, and physical activity using linear mixed effects models on the log scale of hormones. Sodas additionally adjusted for total caffeine intake.
[3] Adjusted for age, waist-to-hip ratio, race, total energy intake, dietary quality score, physical activity, and relevant phase-specific hormone levels using weighted linear mixed effects models on the log scale of hormones with inverse probability of exposure weights. Sodas additionally adjusted for total caffeine intake.
[4] $P<0.05$

β=0.09 [95% CI: 0.03, 0.14]). No significant associations were found between 1-cup increments of artificially sweetened soda or juice intake and reproductive hormones. Nonlinear harmonic models mirrored the linear mixed models between added sugar, fructose, and sweetened soda intake and E2. Women who consumed ≥ 73.2 g/day of added sugar had elevated mean concentrations of free and total E2 concentrations (free E2: β=0.09 [95% CI: 0.02, 0.16]) in addition to increased amplitude for LH (β=0.13 [95% CI: 0.04, 0.21]) after adjusting for age, waist-to-hip ratio, race, total energy intake, dietary quality score, and physical activity (Figures 3.1-3.3). Similarly, women who consumed ≥ 41.5 g/day of fructose had a trend towards elevated free and total E2 (free E2: β=0.05[95% CI: -0.009, 0.11]), and increased amplitude for LH (β=0.06 [95% CI: -0.03, 0.14]), but not statistically significant (Figure 3.1-3.3). In regard to beverage intake, women who consumed ≥ 1 cup/day of sweetened soda had elevated mean free and total E2 levels (free E2: β=0.14 [95% CI: 0.02, 0.26]) after multivariate adjustment (Figure 3.4).

Anovulation

No significant associations were found between intake of added sugar (≥40.0 g/day versus <40.0 g/day or ≥73.2 g/day versus <73.2 g/day), fructose (≥41.5 g/day versus <41.5 g/day), soda, or juice intake (≥1 cup/day vs <1 cup/day) and ovulatory function (Table 3.4). Sensitivity analyses for 1-cup increments of beverage consumption yielded no statistically significant results nor did assessing anovulation based on the less conservative definition (peak progesterone ≤ 5 ng/mL).

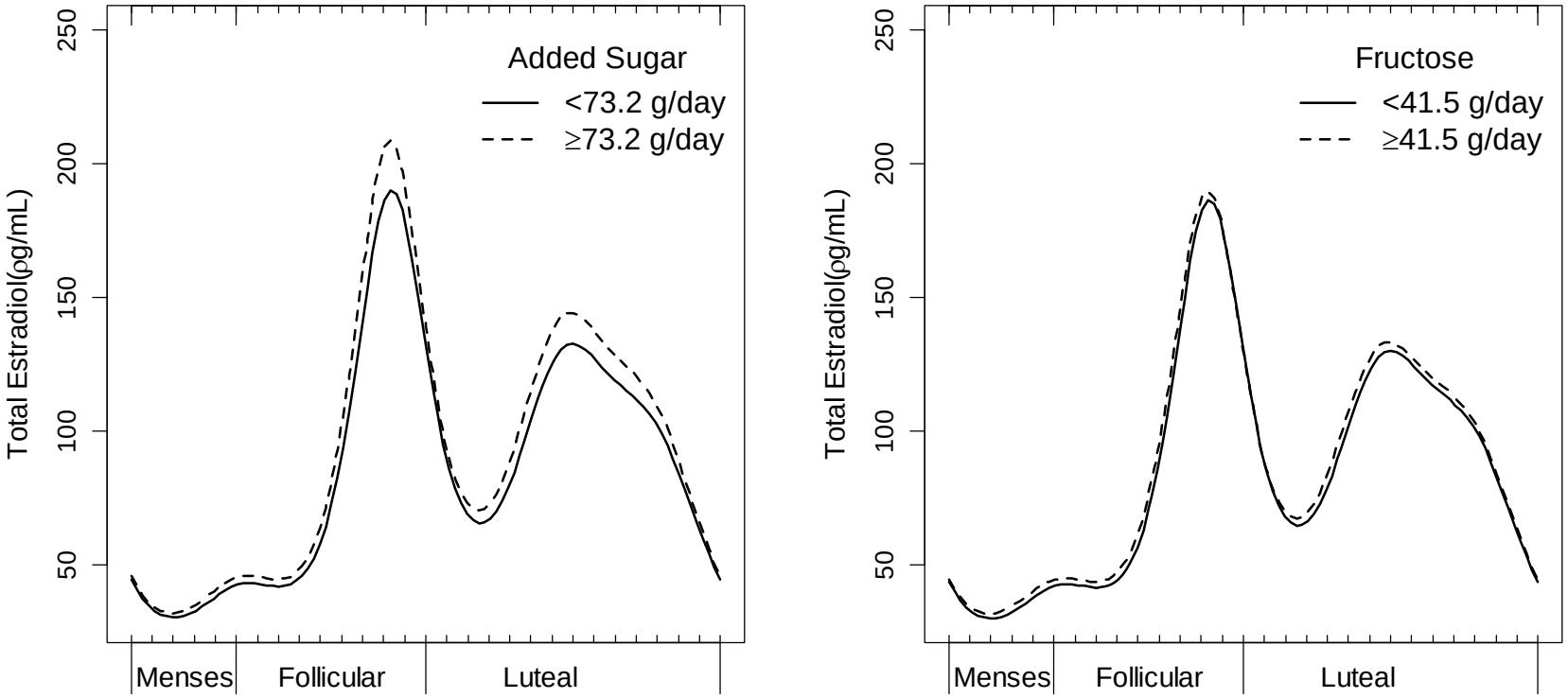

Figure 3.1: Adjusted mean serum total E2 concentrations across the menstrual cycle according to added sugar (≥73.2 g/day vs. less; usual intake for American women) and fructose intake (≥41.5 g/day vs. less; 66th percentile) based on nonlinear harmonic models centered on the day of ovulation (*n*=467 cycles)

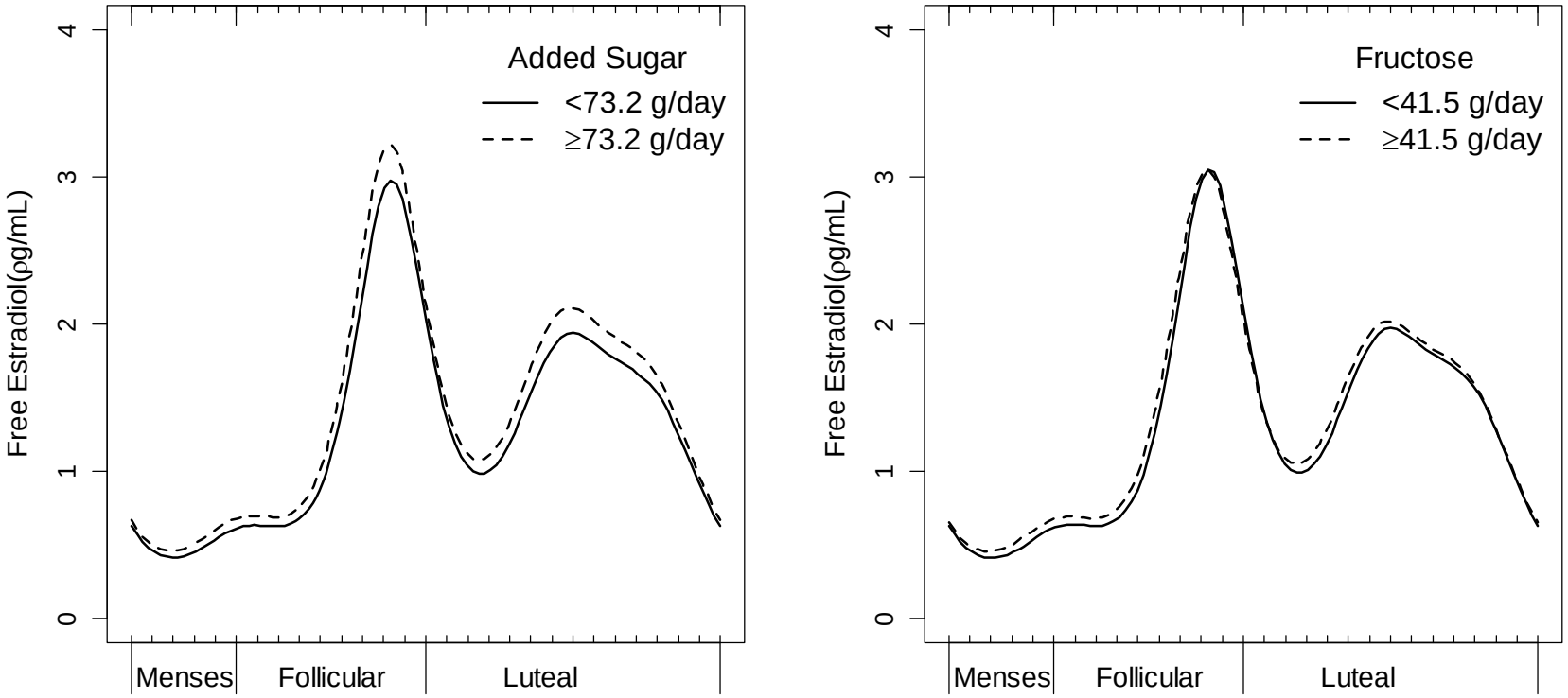

Figure 3.2: Adjusted mean serum free E2 concentrations across the menstrual cycle according to added sugar (≥73.2 g/day vs. less; usual intake for American women) and fructose intake (≥41.5 g/day vs. less; 66th percentile) based on nonlinear harmonic models centered on the day of ovulation (*n*=467 cycles)

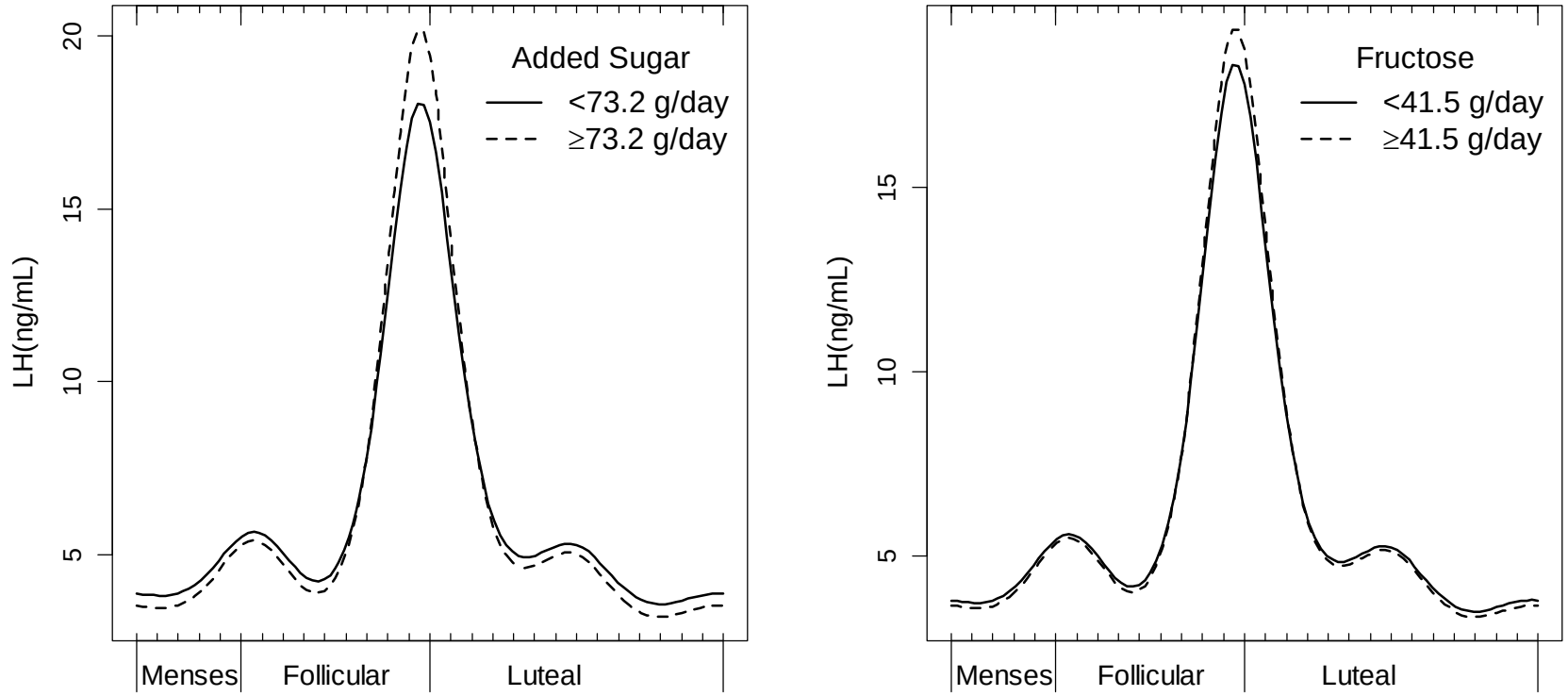

Figure 3.3: Adjusted mean serum LH concentrations across the menstrual cycle according to added sugar (≥73.2 g/day vs. less; Usual intake for American women) and fructose intake (≥41.5 g/day vs. less; 66th percentile) based on nonlinear harmonic models centered on the day of ovulation (*n*=467 cycles)

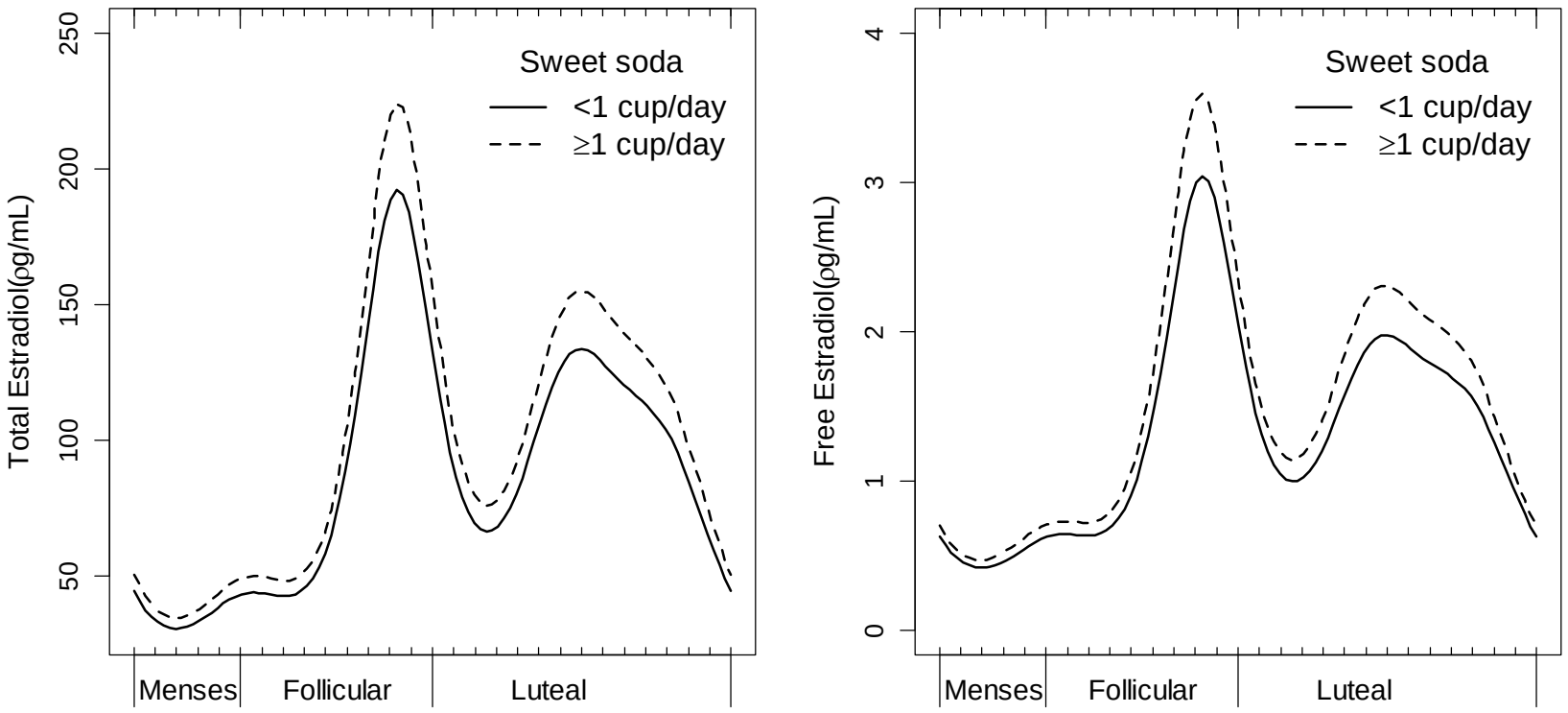

Figure 3.4: Adjusted mean serum total and free E2 concentrations across the menstrual cycle according to sweetened soda intake (≥1 cup/day vs. less) based on nonlinear harmonic models centered on the day of ovulation (*n*=467 cycles)

Table 3.4: Odds of anovulation[1] with consumption of added sugar, fructose and fructose-rich beverages[2]

		Multivariate adjusted OR (95% CI)[3]
Nutrient Intake		
Added Sugar (AHA recommended limit)[6]	**≥ 40 vs < 40 g/day**	0.94 (0.53, 1.64)
Added Sugar	**≥ 73.2 vs < 73.2 g/day**	0.73 (0.37, 1,43)
Fructose	**≥ 41.5 vs < 41.5 g/day**	0.74 (0.39, 1.40)
Beverage Intake		
Sweetened Soda	**≥ 1 vs < 1 cup/day**	1.29 (0.53, 3.14)
Artificially Sweetened Soda	**≥ 1 vs < 1 cup/day**	1.10 (0.35, 3.47)
Cola Soda	**≥ 1 vs < 1 cup/day**	1.00 (0.29, 1.98)
Non-Cola Soda	**≥ 1 vs < 1 cup/day**	0.76 (0.29, 1.97)
Citrus Fruit Juice	**≥ 1 vs < 1 cup/day**	0.79 (0.34, 1.83)
Non-citrus Fruit Juice	**≥ 1 vs < 1 cup/day**	0.67 (0.25, 1.84)

[1]Anovulation is any cycle with peak progesterone concentration ≤ 5 ng/mL and no observed serum LH peak on the mid or late luteal phase visits (*n*=42 cycles).
[2] Intake assessed at 4 times each cycle: menses, mid-follicular, ovulation and mid-luteal clinic visits via 24-hour dietary recall. Fructose equal to the intake of free fructose plus half the intake of sucrose.
[3]Adjusted for age, waist-to-hip ratio, race, total energy intake, dietary quality score, and physical activity using generalized linear mixed models. Sodas additionally adjusted for total caffeine intake.

Discussion

We observed that while added sugar intake above the recommended AHA levels was not associated with reproductive hormone levels, those who consumed above the average intake for American women for added sugars (≥73.2 g/day) or above the 66th percentile for fructose (≥ 41.5 g/day) had elevated free E2, FSH, and LH concentrations compared to women who consumed less. Sweetened soda intake (≥1 cup/day versus < 1 cup/day) was associated with elevated free and total E2 but no statistically significant associations between artificially sweetened soda and fruit juice and reproductive hormones were found. Fructose and fructose-rich beverages were not associated with sporadic anovulation among the BioCycle participants. Findings from this study suggest that intakes greater than typical levels among American women of added sugars, fructose and sweetened sodas may increase serum levels of reproductive hormones but do not interfere with ovulation among healthy premenopausal women with no known ovulatory disorders. Following the AHA guidelines for dietary added sugars appears to be prudent if elevated reproductive hormone concentrations are of concern.

Our finding that added sugar, total fructose, and sweetened soda consumption was significantly and positively associated with reproductive hormone concentrations after multivariable adjustment is novel in humans. While we are aware of no previous animal or human studies investigating the relationship between E2, progesterone, FSH, or LH, and fructose or added sugar, our results showing increased E2 concentrations with sweetened soda intake mirror results from animal studies (36). Celec et al. found intake of 3 different sweetened cola drinks to be associated with increased E2 levels in adult male Wistar rats (*n*=40). The 2 previous studies investigating cola soda intake and

reproductive hormone levels in humans found null associations (14, 15). In particular, Lucero et al. found no association between ≥ 1 cup/day of caffeinated cola intake with geometric mean levels of early follicular phase FSH, LH, total E2 or SHBG concentrations in a study of 498 predominately (97%) white women ages 36 to 45 (15) while Nagata et al. found no significant association between cola and follicular or luteal E2 among Asian college women (*n*=50) in Japan (14). Comparing Lucero's and Nagata's studies with ours is limited since their assessment of diet was retrospective and their hormone measurement included at most 2 serum samples over 1 menstrual cycle, while we assessed diet prospectively and obtained up to 16 serum samples over 2 menstrual cycles using a validated method to time cycle phase (19). Finally, we assessed both cola and noncola sodas. While caramel coloring in cola sodas (which contains advanced glycation end products) has been associated with adverse health effects in animal models (25), we found no distinction in the relationship between cola and noncola sodas with reproductive hormones, indicating that this is not a component of concern regarding reproductive health. Alternatively, given that BioCycle Study participants consumed less cola soda than average US populations, their consumption of such beverages may have been too low to detect any differences between cola and noncola sodas.

While limited data exist on the effect of fructose and sweetened soda on reproductive hormones, other studies have demonstrated that sweetened beverages are associated with impaired fasting glucose and metabolic syndrome (37, 38). Since sucrose and high fructose corn syrup (HFCS) are both composed of roughly equal parts glucose and sucrose, many believe that the effects on the endocrine system are equivalent (1). Others, in contrast, contend that the effects of HFCS-sweetened beverages differ from the

effect of sucrose-sweetened beverages or fructose-rich beverages (e.g., fruit juice) (38), due to the reactive carbonyls found in drinks containing HFCS (39). While we could not distinguish between sodas sweetened with sucrose from those sweetened with HFCS, our results showing elevated reproductive hormone concentrations with added sugars, total fructose, and a variety of sweetened beverages (including an elevated trend with fruit juice) support the hypothesis that the effects of fructose do not differ between sucrose- or HFCS-sweetened beverages.

The question remains, however, as to whether fructose or some other component in sweetened beverages is associated with elevated E2 concentrations. Including fructose intake in our multivariate models with beverages made the relationship between sweetened soda, fruit juice intake, and E2 weaker, suggesting that fructose explains part of the association. While fructose may contribute, the trend towards elevated E2 with artificially sweetened soda intake $\geq$ 1 cup/day suggests that additional components may be at work. Other studies have found that sodas (regardless of sugar content) contribute to adverse health effects, (13, 40) including a recent study by Chavarro et al. that found an increased risk of ovulatory disorder infertility among premenopausal women in the Nurses' Health Study for both sweetened and artificially sweetened soda (13). The theory that soda (irrespective of sugar content) may be replacing a nutrient or food component in the diet, and that it is the lack of this component causing the effect is well documented in the literature (41). Individuals who consume soda are known to also have greater caloric and fat intake (42), and a more sedentary lifestyle (43). Soda consumers in the BioCycle Study were more likely to have a higher waist-to-hip ratio, a lower percent of calories from protein intake, and be less physically active. Indeed, after adjusting for diet and

adiposity as well as total energy intake, we found no statistically significant associations between artificially sweetened soda and reproductive hormones; however, the associations we observed between added sugar, fructose, and sweetened sodas remained. While residual confounding by either lifestyle or socio-economic factors may persist, our well-measured prospective assessment of diet and physical activity may explain why we, as well as others (44), have found differing effects between sweetened and artificially sweetened sodas compared to others who have not (13, 40). Additionally, although fruit juices are a known contributor to fructose intake, our finding of a statistically significant association between sweetened soda and reproductive hormone concentrations, but not fruit juice, may be due to the beneficial components of fruit juice including vitamins and antioxidants (44), which are often lacking in consumers of sugar-sweetened beverages (45). Further research looking at the relationship between different juice types (including freshness and processing (44)) and premenopausal reproductive hormones is needed.

The BioCycle Study has several strengths, including multiple measures of hormone assessment over 2 menstrual cycles (using standardized methods to time cycle phase) and multiple measures of not only fructose and fructose-rich beverage intake, but important dietary and lifestyle factors as well. While self-report of diet is subject to measurement error (46-48), our study used multiple validated 24HDRs to reduce the potential for misclassification in added sugar, fructose and beverage exposure. Additionally, we assessed recall validity by comparing total soda intake with the averages obtained from 2 food frequency questionnaires captured over the same time period and found significant correlation (r=0.70). Nevertheless, the study was limited by the relatively low consumption of added sugar and soda intake. US premenopausal women

consume on average 78 g/day of added sugar, 19 oz of soda, and 3 oz of fruit juice (2) whereas the BioCycle Study participants consumed on average 57.2 g/day of added sugars, 3 oz of soda, and 4 oz of fruit juice.

In conclusion, mean intake of added sugars greater than the intake of the average American woman (≥ 73.2 grams/day), fructose ≥ 41.5 g/day, and sweetened soda ≥ 1 cup/day are associated with elevated E2 concentrations. While we observed no effect on incident anovulation with moderate sweetened beverage consumption, further research investigating higher consumption (i.e., around the US female average of ≥ 2 8-oz servings/day), along with inclusion of women with more pronounced ovulatory disorders is warranted (13). Since a randomized trial investigating the effects of soda on reproductive function may not be feasible, further methodologically rigorous observational studies using gold-standard measures of exposure assessment are needed to better understand the effects of fructose on reproductive hormone levels and ovulatory function. While recent research indicates that consumption of added sugars is decreasing in the Unites States (49), mean intakes among premenopausal women continue to exceed recommendations. Our findings have public health implications not only for the role that fructose and fructose-rich beverages have on female fertility, but also for their potential relationships with a woman's future risk for chronic diseases associated with reproductive hormones.

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CHAPTER 4

CAFFEINE VALIDATION USING DIFFERENT INSTRUMENTS IN THE BIOCYCLE STUDY

Abstract

The effects of caffeine on women's health are inconclusive due in part to inadequate exposure assessment. We determined validity of a food frequency questionnaire (FFQ) for measuring monthly caffeine and caffeinated beverage intake compared to multiple 24-hour dietary recalls (24HDR); and validity of the 24HDR for measuring daily caffeinated coffee intake compared to prior days' diary record. The BioCycle Study (2005-2007) prospective cohort (n=259) included women, ages 18-44, who were followed for 2 menstrual cycles, completing up to 4 24HDRs per cycle, an FFQ at the end of each cycle, and daily diaries. Caffeine was analyzed using the Nutrition Data System for Research (2005) for the FFQ and 24HDR. We determined validity of mean caffeine and caffeinated beverage intake from the FFQs compared to the 24HDRs via Wilcoxon's matched-pairs, signed-ranks tests and Pearson's correlation coefficients. We assessed agreement via cross-classification analyses and kappa statistics. Repeated measures analyses evaluated validity of the 24HDR compared to the daily diary. Caffeine intake reported in the FFQ was greater than that reported in the 24HDRs (mean=114.1 versus 92.6 mg/day; P=0.006) despite high correlation (r=0.80, P< 0.001) and moderate agreement (kappa=0.56, 95% Confidence Interval: 0.42-0.70). Women reported greater

caffeinated coffee intake in their daily diary compared to their corresponding 24HDR (mean=0.80 versus 0.51 cups/day, $P< 0.001$). Although caffeine and coffee exposures were highly correlated, absolute intakes differed significantly between measurement tools. These results highlight the importance of considering potential misclassification of caffeine exposure. Further validation with biomarker assessment is needed.

Introduction

Caffeine, the most widely consumed drug in the world (1), is ingested primarily through coffee, tea, and soda and has received a great deal of attention regarding its health effects on premenopausal women (2, 3). Coffee, tea, and soda contain other components in addition to caffeine that may affect health, highlighting the importance of beverage source (4, 5). Health effects of caffeine and caffeinated beverages have been inconclusive due in part to inadequate exposure assessment (2, 3).

Measuring caffeine intake is difficult since it occurs in a variety of sources (6). Additionally, caffeine exposure can vary depending on brand, serving size, and method of preparation (7). Retrospective assessment of caffeine at a single time point may be prone to measurement error since it fails to account for exposure fluctuations (2) and prospective assessment may lack precision if it fails to capture the caffeine content of different foods and beverages (8). Caffeine exposure misclassification may bias effect estimates towards or away from the null depending on the magnitude and direction of the errors (2). Therefore, it is important to determine the validity of common methods of measuring caffeine consumption among reproductive-aged women.

Most studies assessing caffeine among nonpregnant, premenopausal women use self-administered, semiquantitative food frequency questionnaires (FFQ) (5, 9-12). Diet

records and recalls are generally considered to be the gold standard for dietary assessment, and thus are often used as the reference when assessing the relationship between reported intakes from an FFQ and true usual intake (13). Various versions of the FFQ have been validated for caffeine intake among non-American, older women in 2 previous studies (mean age: 54 and 58 years) (13, 14); and for caffeinated beverages (i.e., coffee, tea, and soda) among pre- to perimenopausal American women (ages 34-59, uniformly distributed) (15). No study to date, however, has investigated the validity of an FFQ for both caffeine and caffeinated beverage intake (caffeine/beverage intake) for American reproductive-age women using appropriate statistical methods. Validation studies depending on correlation analyses alone are inadequate since correlation measures the strength of the linear relationship, not agreement, and correlations depend on the range of the true quantity within the sample (16).

Our primary objective was to assess the validity of 1) the FFQ for measuring monthly caffeine/beverage intake compared to multiple 24-hour dietary recalls (24HDRs) and 2) the 24HDR for measuring daily caffeinated coffee intake compared to daily diary (DD) records. Our secondary objective was to assess the variability of caffeine consumption patterns by comparing 1) caffeine/beverage intake for 8 24HDRs and (for caffeinated coffee) DDs, captured over 2 menstrual cycles, and 2) caffeine/beverage intake for the FFQ completed at baseline (capturing the previous 6 month's intake) with the FFQ completed at the end of each menstrual cycle (capturing the previous cycle's intake).

Subjects and Methods

Study Population

Women, ages 18-44, were recruited between 2005-2007 from western New York State and enrolled for 1 (*n*=9) or 2 (*n*=250) menstrual cycles in the BioCycle Study. The study population, materials and methods have been previously described in detail (17). In summary, eligible women had to be healthy, with self-reported cycle length from 21-35 days for the previous 6 months, no use of hormonal contraception for the past 3 months, and without known conditions that affect the menstrual cycle, such as polycystic ovary syndrome or uterine fibroids. Physical measures were obtained in the clinic using standardized protocols and socio-demographic and lifestyle information was collected using validated questionnaires (17). The University at Buffalo Health Sciences Institutional Review Board (IRB) approved the study and served as the IRB designated by the National Institutes of Health for this study under a reliance agreement. All participants provided written informed consent.

Dietary Assessment

Food Frequency Questionnaire (FFQ)

Participants completed an FFQ up to 3 times at the clinic; once at baseline (FFQ-B) to capture usual intake for the previous 6 months and during the late luteal phase of each cycle to determine usual intake in the month of each cycle (FFQ-1 and FFQ-2). Nutrient data were collected using the FFQ developed by the Nutrition Assessment Shared Resource (NASR) of the Fred Hutchinson Cancer Research Center (FHCRC), which calculated nutrient intakes using the Nutrition Data System for Research (NDSR) software (version 2005), developed by the Nutrition Coordinating Center, University of

Minnesota, Minneapolis, MN. NDSR uses the standardized, multiple-pass approach of interview methodology, and computes the nutrients (e.g., caffeine in mg/day) and the food/beverage components (e.g., unsweetened coffee in cups/day) from the assessments. The NDSR calculates caffeine intake based on consumption-weighted averages of values provided by the USDA database. The BioCycle Study used the general FFQ (GSEL). This self-administered FFQ asks participants to report on the frequency of consumption (e.g., never or less than once per month to 6+ per day) and portion size (e.g., small, medium, or large with medium size described) of approximately 120 line items, including 5 caffeinated beverages. Ninety-nine percent of the participants completed at least 1 of the FFQs, while 86% completed all 3 FFQs (FFQ-B, FFQ-1, and FFQ-2).

24-Hour Dietary Recall (24HDR)

Participants completed a 24HDR at the clinic after fasting blood specimen collection during the visits corresponding to menstruation, mid-follicular phase, ovulation, and mid-luteal phase. Information regarding food and beverage intake was collected and nutrient data were analyzed using NDSR (version 2005). Seventy-three percent of participants completed all 8 24HDRs, 96% completed 4 24HDRs in at least 1 of their cycles under study, while 99% completed at least 3 24HDRs per cycle (i.e., 249 out of the 250 women contributing 2 cycles completed at least 3 24HDRs in both of their cycles while all 9 of the women contributing 1 cycle completed at least 3 24HDRs for that cycle).

Daily Diary (DD)

Participants recorded daily caffeinated coffee intake and other lifestyle/health items on DD forms. Study staff instructed participants to begin completing their DD on the first day of their next menstrual period and continue daily through the next 2 menstrual cycles. Participants recorded the number of 8 oz cups (hot or iced/instant or brewed) of caffeinated coffee consumed daily. Ninety-seven percent of participants completed at least 75% of the DDs in at least 1 of their cycles; 71% of participants completed 100% (i.e., no missed days) in at least 1 of their cycles.

Statistical Analysis

Validity of Caffeine/Beverage Intake

Descriptive statistics were calculated including socio-demographic characteristics. Caffeine/beverage intake from the 24HDRs and FFQs were non-normally distributed and therefore nonparametric analysis techniques were used. To determine the validity of the FFQ compared to the 24HDR, women who completed either the FFQ-1 or FFQ-2 and at least 75% of their 24HDRs for the corresponding cycle were included in the analyses (n=249). To validate the DD compared to the 24HDR, women who completed at least 75% of their 24HDRs and DDs in at least 1 of their cycles (n=251) were included.

To evaluate validity of the FFQ for assessing monthly caffeine/beverage intake, we compared the mean value of FFQ-1 and FFQ-2 with the mean value of the 8 24HDRs. We additionally compared the 4 24HDRs per cycle with their corresponding FFQ. We report means and standard deviations (SD) along with medians and interquartile ranges (IQR) of daily caffeine/beverage intake from the FFQs and 24HDRs, and used the

Wilcoxon's matched-pairs signed-ranks test to determine differences between the means. Pearson's correlation coefficients on log-transformed values described the associations between the FFQs and 24HDRs. We also calculated deattenuated Pearson's correlation coefficients where the within-woman variations were divided by the between-woman variations to quantify the variance ratios of the 24HDRs (18).

To visualize agreement between the FFQ and 24HDR for caffeine/beverage intake, we constructed Bland and Altman plots using the mean value of FFQ-1 and FFQ-2 and the 8 24HDRs. We present the plots on the original scale with back-transformed limits of agreement (LA) (19). To evaluate the FFQ's ability to assign women to the same categories of intake as the 24HDR, women were classified into tertile categories of caffeine/beverage intake based on the distribution of data from both the FFQ and 24HDR (20, 21). Due to the highly skewed data for coffee drinks/cocoa, cut points at the 10^{th} and 90^{th} percentile were used to create the categories for coffee drinks/cocoa. We performed cross-classification analyses and compared percent agreement and weighted κ coefficients calculated with a linear set of weights in addition to calculating actual values for surrogate tertiles of caffeine/beverage intake (cutpoints of 10^{th} and 90^{th} percentile for coffee drinks/cocoa) with the FFQ and the 24HDR as the surrogate and reference method, respectively (20). We used recommended levels of daily caffeine intake (preconception counseling: $\leq$200 mg/day) (22) as the threshold value to estimate specificity, sensitivity, and positive and negative predictive values of the FFQ, whereby intakes in line with the recommended levels were defined as positive.

To evaluate validity of the 24HDR for assessing daily caffeinated coffee intake, we used the above analyses to compare the mean caffeine intake for the 24HDRs with the

previous day's DD. We chose a relevant cut point (≥ 1 cup/day versus < 1cup/day) (12) to estimate specificity, sensitivity, and positive and negative predictive values of the 24HDR.

Variability of Caffeine/Beverage Intake

To determine whether there was a habitual pattern of caffeine/beverage intake over the study period as reported in the FFQ, we repeated the above analyses to assess the agreement between FFQ-1 and FFQ-2 (with the exception of de-attenuating the correlation coefficients, deemed unnecessary for reproducibility studies) (20), restricting to women who completed both FFQ-1 and FFQ-2 (*n*=224). We compared FFQ-B with the mean of FFQ-1 and FFQ-2, to account for changes in consumption while under observation, restricting to women who completed all 3 FFQs (*n*=222).

To determine habitual pattern of caffeine/beverage intake as reported in the 24HDRs, we used repeated measures analyses with random intercepts, restricting to women who completed at least 75% of their 24HDRs for a given cycle (*n*=258). These models accounted for between-woman variation in baseline caffeine intake and within-woman correlation. *P* values correspond to 2-sided tests with significance set at 0.05. Analyses were performed in SAS version 9.2 (SAS Institute, Cary, NC).

Results

Population Characteristics

The mean age of participants included in the primary validity study (*n*=249) was 27.5 (*SD*=8.3). Participants were of normal weight (mean BMI of 24.1 (*SD*=3.8)), predominately white (59.2%), currently nonsmokers (defined as no current cigarette use

as recorded in their daily diaries) (95.6%), and nulligravidous (69.1%). Demographics of women included in variability analyses as assessed by the FFQ (*n*=224) were similar to the validity study.

Validity of Caffeine/Beverage Intake

According to FFQ-B, 58% reported consuming coffee, 72% tea, 64% lattes, cappuccinos, mochas, or hot chocolate (coffee drinks/cocoa), and 77% soda. Similar patterns were seen for the FFQ-1 and FFQ-2, with 60% consuming coffee, 72% tea, 65% coffee drinks/cocoa, and 80% soda. Average 24HDR beverage consumption was less than that reported by FFQs, with 49% consuming coffee, 64% tea, 21% coffee drinks/cocoa, and 71% soda.

Compared to the 24HDR, the FFQ significantly overestimated usual daily caffeine (mean=114.1 versus 92.6 mg/day; geometric mean=48.9 versus 41.4; P=0.005), coffee (mean=0.76 versus 0.51 cups/day; geometric mean=0.11 versus 0.08; P<0.001), and coffee drinks/cocoa intake (mean=0.18 versus 0.09 cups/day; geometric mean=0.05 versus 0.02; P<0.001); and underestimated usual daily soda intake (mean=0.41 versus 0.57 cups/day; geometric mean=0.12 versus 0.16; P<0.001), although the log-transformed caffeine/beverage intakes were all significantly correlated (P<0.001) (Table 4.1). Despite divergence, the Bland-Altman plots showed acceptable relative limits of agreement (Figure 4.1). The intrawoman LA were ± 1.14 for caffeine, ± 0.94 for coffee, ± 1.34 for coffee drinks/cocoa, ± 1.45 for tea, and ± 1.24 for soda. Differences for all beverages followed a normal distribution except for coffee. Results were similar when we compared the average of the 24HDRs per cycle with the corresponding FFQ (data not shown).

Table 4.1: Usual daily intakes of caffeine and caffeine-related beverages calculated from the FFQ and 24HDR (*n*=249); differences and correlation coefficients between the mean FFQ (test method) and 24HDR (reference method)[1]

	FFQ		24HDR		*P* [2]	Correlation	
	Mean ± *SD*	Median (IQR)	Mean ± *SD*	Median (IQR)		*R*[3]	*R*[4]
Caffeine (mg/day)	114.1 ± 146.1	68.1 (19.5-147.5)	92.6 ± 95.1	59.8 (19.4-140.8)	0.006	0.68	0.73
Coffee (cups/day)	0.76 ± 1.34	0.09 (0.00-1.00)	0.51 ± 0.75	0.00 (0.00-0.94)	<0.001	0.91	0.99
Coffee drinks/cocoa (cups/day)	0.81 ± 0.50	0.05 (0.00-0.15)	0.09 ± 0.33	0.00 (0.00-0.00)	<0.001	0.39	0.40
Tea (cups/day)	0.38 ± 0.75	0.09 (0.00-0.39)	0.36 ± 0.49	0.17 (0.00-0.50)	0.38	0.57	0.59
Soda (cups/day)	0.41 ± 0.68	0.12 (0.03-0.42)	0.57 ± 0.71	0.31 (0.00-0.80)	<0.001	0.68	0.71

[1] 24HDR, 24-hour dietary recall, average of 8 24HDRs over 2 cycles; FFQ, food-frequency questionnaire, average of FFQ1 and FFQ2 over 2 cycles. Coffee is all types "not lattes or mochas"; coffee drinks/cocoa includes "latte, cappuccino, mocha or hot chocolate"; "tea is "all types"; and soda includes diet and regular soft drinks.

[2] Wilcoxon's matched-pairs signed-rank test

[3] Pearson's correlation coefficient on log-transformed data. All correlations significant at $P<0.001$.

[4] Pearson's deattenuated correlation coefficient on log-transformed data.

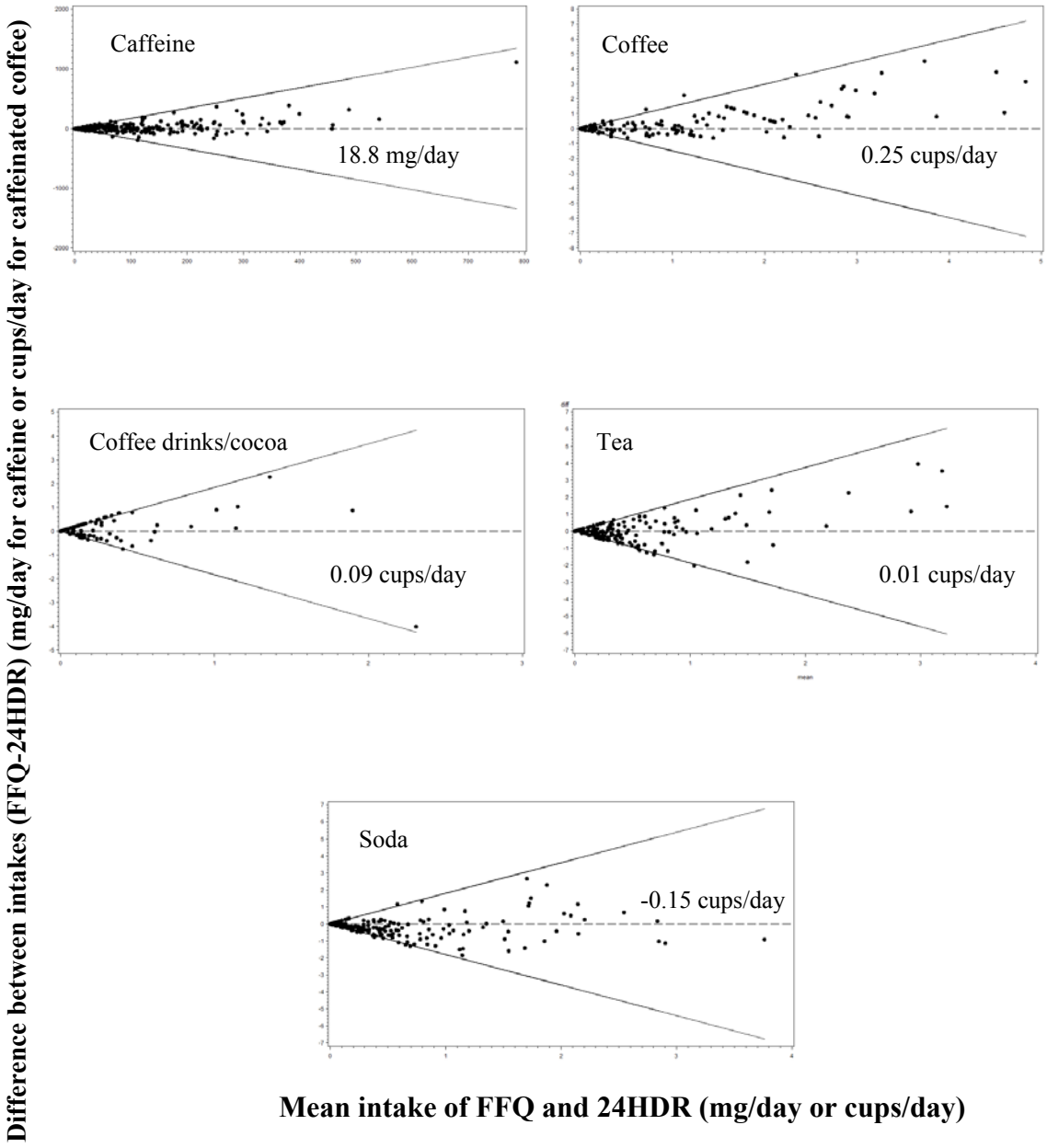

Figure 4.1: Bland-Altman plots of difference in caffeine (mgs/day) and beverage (cups/day) intakes between the FFQ and 24HDR. Dotted line in each graph represents mean difference between FFQ and 24HDR on original scale; solid lines represent relative limits of agreement from the logarithmic scale

The majority (55–79%) of women were assigned to the same tertiles by both methods except for coffee drinks/cocoa where the majority of women (49%) were assigned to the adjacent, 45% to the same, and 6% to the extreme category (Table 4.2). Weighted κ values showed substantial agreement for coffee; moderate agreement for caffeine, soda, and tea; and slight agreement for coffee drinks/cocoa. Actual values for surrogate tertiles of usual daily caffeinated beverage intake are shown in Table 4.3. Using recommended daily amounts for caffeine as the threshold value (<200mg/day), sensitivity of the FFQ was 0.90 while specificity was 0.79. The positive and negative predictive values were 0.97 and 0.56.

Despite high correlation (Pearson's correlation coefficient on log-transformed data=0.77, $P<0.001$), women reported significantly less caffeinated coffee intake in the 24HDR compared to the corresponding day's DD (mean=0.51 versus 0.80 cups/day; geometric mean=0.05 versus 0.08; $P< 0.001$) (Figure 4.2). Mean differences between the 24HDR and the DD were similar for both cycles.

Variability of Caffeine/Beverage Intake

Mean daily intakes of caffeine and caffeinated beverages for FFQ-1 and FFQ-2 were highly correlated (0.72 to 0.94) as were intakes for FFQ-B and FFQ-1&2 (0.76 to 0.94) (Table 4.4). While no statistically significant differences were found in mean daily intakes between FFQ-1 and FFQ-2, coffee intake was lower in FFQ-B compared with FFQ-1&2 (mean=0.69 to 0.77 cups/day, geometric mean=0.10 versus 0.11; P=0.02) while tea intake was higher in FFQ-B (0.47 to 0.38 cups/day; geometric mean=0.10 versus 0.09; P=0.04). Cross-classification between FFQ-1 and FFQ-2 showed little severe misclassification and substantial agreement for caffeine, coffee, and soda tertiles

Table 4.2: Cross-classification and κ coefficient of the FFQ and 24HDR tertiles[1] of daily caffeine, coffee, coffee drinks/cocoa, tea and soda intakes (*n*=249 women)

	Same Category *n* (%)	Adjacent Category *n* (%)	Extreme Category *n* (%)	Weighted κ (95% CI)
Caffeine (mg/day)	159 (64)	80 (32)	10 (0.04)	0.55 (0.47, 0.64)
Coffee (cups/day)	196 (79)	50 (20)	3 (0.01)	0.76 (0.70, 0.82)
Coffee drinks/cocoa (cups/day)	111 (45)	123 (49)	15 (6)	0.20 (0.12, 0.27)
Tea (cups/day)	136 (55)	95 (38)	18 (7)	0.41 (0.31, 0.50)
Soda (cups/day)	153 (61)	86 (35)	10 (4)	0.51 (0.43, 0.60)

[1]Due to the highly skewed data for coffee drinks/cocoa, cut points were at 10th and 90th percentiles.

Table 4.3: Actual values for surrogate tertiles[1] of usual daily caffeine, coffee, coffee drinks/cocoa, tea and soda intake with the FFQs and the 24HDRs as the surrogate and reference method, respectively

	1st tertile Mean ± *SD*	2nd tertile Mean ± *SD*	3rd tertile Mean ± *SD*	*P*[2]
Caffeine (mg/day)	36.2 ± 46.9	68.9 ± 50.2	190.7 ± 101.8	<0.001
Coffee (cups/day)	0.0 ± 0.1	0.2 ± 0.3	1.4 ± 0.7	<0.001
Coffee drinks/cocoa (cups/day)	0.0 ± 0.1	0.1 ± 0.4	0.3 ± 0.4	<0.001
Tea (cups/day)	0.2 ± 0.3	0.3 ± 0.4	0.6 ± 0.6	<0.001
Soda (cups/day)	0.2 ± 0.3	0.4 ± 0.4	1.2 ± 0.8	<0.001

[1]Due to the highly skewed data for coffee drinks/cocoa, cut points were at 10th and 90th percentiles.
[2]Kruskal-Wallis test

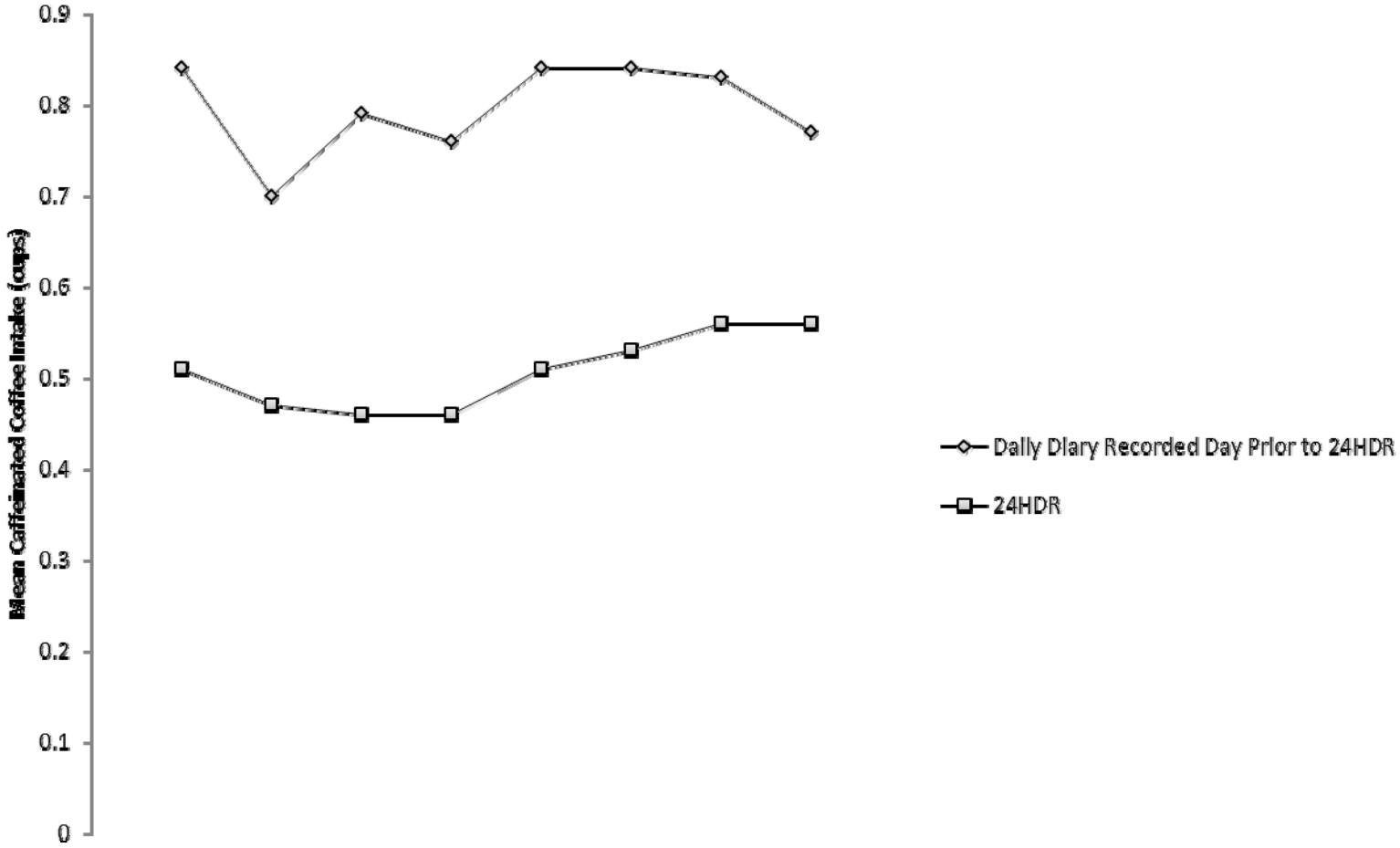

Figure 4.2: Usual daily intakes of cups of caffeinated coffee calculated from the 24HDR and DD' (*n*=258 women) comparing caffeinated coffee intake reported in the diary on the day preceding the clinic visit and with that reported in the 24HDR at the clinic visit over the 2 cycles

Table 4.4: Usual daily intakes of caffeine and caffeinated beverages calculated from FFQ; differences and correlation coefficients between FFQs[1]

	FFQ-1		FFQ-2		P^2	Correlation R^3
	Mean ± *SD*	Median (IQR)	Mean ± *SD*	Median (IQR)		
Caffeine (mg/day)	112.6 ± 130.8	68.7 (16.4-157.2)	114.5 ± 136.3	71.5 (15.6-153.0)	0.92	0.86
Coffee (cups/day)	0.75 ± 1.27	0.06 (0.00-1.00)	0.79 ± 1.37	0.06 (0.00-1.00)	0.34	0.94
Coffee drinks/cocoa (cups/day)	0.15 ± 0.32	0.06 (0.00-0.14)	0.15 ± 0.39	0.02 (0.00-0.14)	0.48	0.72
Tea (cups/day)	0.39 ± 0.79	0.06 (0.00-0.39)	0.37 ± 0.84	0.06 (0.00-0.39)	0.10	0.76
Soda (cups/day)	0.42 ± 0.78	0.12 (0.03-0.39)	0.41 ± 0.74	0.12 (0.03-0.39)	0.86	0.84
	FFQ-B		**FFQ-1&2**		P^2	Correlation R^3
	Mean ± *SD*	Median (IQR)	Mean ± *SD*	Median (IQR)		
Caffeine (mg/day)	114.5 ± 140.4	71.8 (20.6-150.8)	113.4 ± 128.6	70.9 (17.6-152.1)	0.66	0.86
Coffee (cups/day)	0.69 ± 1.21	0.06 (0.00-1.00)	0.77 ± 1.28	0.10 (0.00-1.00)	0.02	0.94
Coffee drinks/cocoa (cups/day)	0.19 ± 0.59	0.06 (0.00-0.14)	0.15 ± 0.32	0.03 (0.00-0.14)	0.15	0.79
Tea (cups/day)	0.47 ± 1.01	0.09 (0.00-0.39)	0.38 ± 0.76	0.09 (0.00-0.39)	0.04	0.76
Soda (cups/day)	0.46 ± 0.82	0.14 (0.03-0.39)	0.41 ± 0.69	0.12 (0.03-0.45)	0.30	0.82

[1]FFQ-1, food-frequency questionnaire captured at end of cycle 1; FFQ-2, food-frequency questionnaire captured at end of cycle 2; FFQ-B, food-frequency questionnaire captured at baseline, FFQ-1&2, average of FFQ-1 and FFQ-2 over 2 cycles. n=224 women for comparison between FFQ-1 and FFQ-2; n=222 women for comparison between FFQ-B and FFQ-1&2.

[2] Wilcoxon's matched-pairs signed-rank test.

[3] Pearson's correlations on log-transformed data.

(weighted κ =0.80, 0.86, 0.71, respectively) (Table 4.5). Coffee drinks/cocoa (κ =0.58) and tea (κ =0.61) showed moderate agreement. Similar levels of agreement were found between FFQ-B and FFQ-1&2.

Neither caffeine nor caffeinated beverage intake as measured by the 24HDR varied significantly over the 2 menstrual cycles (Figure 4.3). Caffeine consumption reported in the DD was also consistent across the cycle (Figure 4.3).

Discussion

We show that although caffeine and caffeinated beverage intake are highly correlated between measurement tools in the BioCycle Study, absolute intakes differed significantly. While the FFQ is appropriate for ranking caffeine and caffeinated beverage exposure, it may not appropriately classify exposure based on clinically relevant cut points. As such cut points are used to guide policy decisions; our findings have broad public health implications. We demonstrate that the FFQ reported caffeine intake was consistent over the 2 menstrual cycles under study; or from the consumption over the previous 6 months, as reported at baseline, to that while under observation. Our analysis of 24HDR and DD reported caffeine/beverage intakes further support that caffeine intake was habitual and relatively consistent over the course of the menstrual cycle. FFQ and 24HDR reported caffeine/beverage intakes were more highly correlated than previous validation studies. Prior population-based studies of women demonstrated deattenuated correlations between 0.64 to 0.76 (13-15 , 23). Given that correlations above 0.50 between a dietary instrument (such as the FFQ) and a reference method (such as a dietary record or 24HDR) indicate that the instrument can reliably rank persons (20), both our and previous studies support the FFQ as a valid instrument to rank intake. The FFQ's

Table 4.5: Cross-classification and κ coefficient of the FFQ-1 and FFQ-2 tertiles of daily caffeine and caffeinated beverage intakes (*n*=224 women).

	Same Category *n* (%)	Adjacent Category *n* (%)	Extreme Category *n* (%)	Weighted κ (95% CI)
Caffeine (mg/day)	177 (79)	37 (17)	10 (4)	0.80 (0.73-0.86)
Coffee (cups/day)	194 (87)	30 (13)	0 (0)	0.86 (0.81-0.91)
Coffee drinks/cocoa (cups/day)	151 (67)	52 (23)	21 (9)	0.58 (0.48-0.66)
Tea (cups/day)	155 (69)	58 (26)	11 (5)	0.61 (0.53-0.69)
Soda (cups/day)	171 (76)	52 (23)	1 (0.004)	0.71 (0.64-0.79)

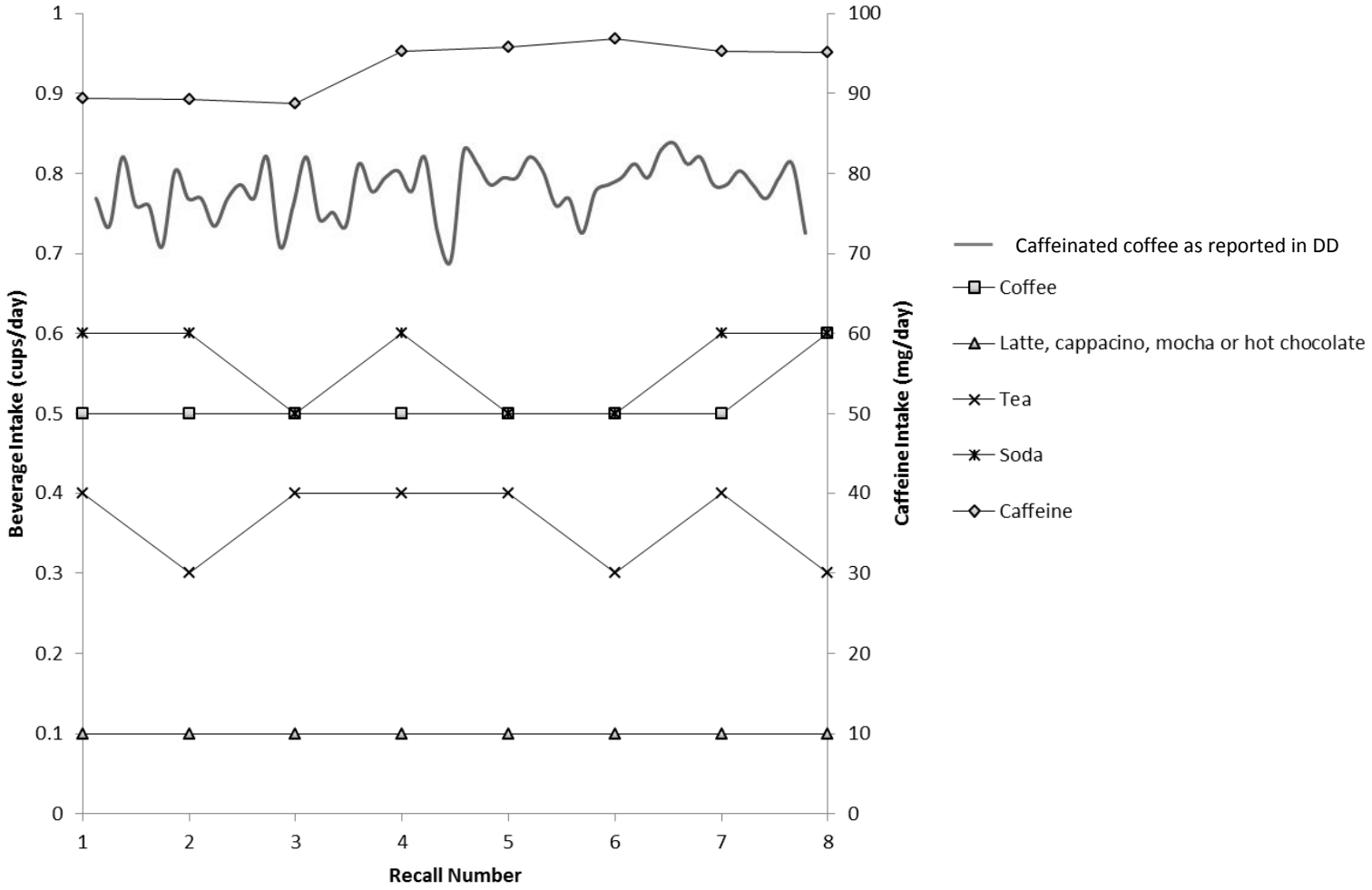

Figure 4.3: Usual daily intakes of caffeine and caffeinated beverages calculated from 8 24HDRs[1] and DDs (*n*=258 women).

ability to rank individuals for caffeinated beverages is not surprising as participants more easily report frequently consumed foods and beverages (20).

While adequate ranking of individuals may be sufficient for many epidemiological analyses (14, 15), assessments of absolute intakes are necessary for formulating recommended levels of consumption and comparability between studies (15). We found that mean caffeine intake reported in the 24HDRs was lower than that reported in the FFQs over the same time period. Our findings agree with comparisons between a dietary record and the FFQ in the Nurses' Health Study for coffee intake (1.8 versus 2.4 cups/day, respectively) (15); but other studies reported higher mean caffeine intake in the 7-day diet record compared to the FFQ (206 versus 143 mg/day) (14), or roughly equivalent caffeine intake between 3 24HDRs and an FFQ corresponding to the same time period (218 versus 216 mg/day)(13).The difference in reported intake between the 24HDR and FFQ could be due to daily variation in consumption patterns. The majority (93%) of 24HDRs in our study occurred on weekdays and, among this population of women, caffeine intake may occur more frequently on weekends, particularly since caffeine and alcohol intake were associated in the BioCycle Study and alcohol consumption is higher on the weekends. Over the 2 menstrual cycles, mean caffeinated coffee intake reported in the DD was equivalent to mean coffee intake reported in the FFQ, suggesting that the 24HDRs may have missed higher coffee consumption days.

The difference in absolute intakes between the FFQ, 24HDR, and the DD may be attributable to the tendency to over-report socially desirable foods and beverages and under-report less healthy foods (15). The standardized, multipass method of a 24HDR may correct for this bias compared to a self-administered FFQ or the DD. Since

participants were instructed to report total number of cups of caffeinated coffee consumed in their DD, rounding up of caffeinated coffee intake may have occurred. Coffee has been publicized to contain antioxidants and chemo-preventive properties, which could account for the statistically significant higher report of coffee intake. Negative reports on soda may explain our finding a significantly lower reported consumption in the FFQ compared to the 24HDR.

Classification analyses for caffeine have been conducted in 1 other study with nearly identical results (weighted κ =0.64), despite a difference in mean caffeine intake in the FFQ between their study (143 ± 105mg) and our study (114 ± 128mg) (14). We found that the FFQ reliably distinguished extreme caffeine intake as documented previously (14). No other studies have assessed actual values for surrogate categories nor looked at the sensitivity, specificity, and positive and negative predictors of the FFQ for caffeine intake based on recommended limits of intake. Use of actual values obtained from the 24HDR for the surrogate categories derived from the FFQ in our study indicated that variation between-women for caffeine and caffeinated beverages is relatively high compared to the relatively low within-woman variation. In terms of how well the FFQ can "screen" women based on recommended levels of intake (22), if we assume that the 24HDR accurately assessed intake, use of the FFQ would wrongly categorize 3% of women below recommended levels and 8% of women above recommended levels.

The FFQ showed that caffeine/beverage intake did not significantly vary for BioCycle Study participants, both between the baseline and study period values (maximal misclassification was 4% for tea) as well as over the course of the study (maximal misclassification was 9% for coffee drinks/cocoa). This indicates that caffeine/beverage

intake among the BioCycle Study participants is neither prone to month-to-month variability nor influenced by enrollment in the study.

Ours is the first study to investigate the validity of the FFQ for reporting of coffee drinks/cocoa. Analyses of specific foods or beverages (e.g., coffee drinks), instead of nutrients (e.g., caffeine), are useful for detecting questionnaire weaknesses and potential modifications (15). Average coffee drinks/cocoa intake between the FFQ and 24HDR were weakly correlated, possibly indicating that the FFQ poorly measures these beverages, as multiple beverages are collapsed into 1 category and showed low between-person variability. If the research aim is to assess caffeine intake, this category should be divided into drinks containing espresso (including number of shots) versus cocoa, given the difference in caffeine content between espresso and chocolate. Such a questionnaire could be validated among premenopausal women to improve assessment of coffee drinks/cocoa for future studies wishing to use an FFQ to assess the effect of caffeine on women's health.

Assessing caffeine by self-report is difficult, due to the variability in caffeine beverage content (24). Available statistical methods to assess usual intake from the 24HDR with supplemental demographic and FFQ information (25) do not address the heterogeneity of caffeine content in beverages nor the between-woman variation in caffeine metabolism. While overall caffeine/beverage intake did not vary over the menstrual cycle (25), within-woman caffeine metabolism may change over the menstrual cycle (26). To improve caffeine exposure assessment among premenopausal women, future studies using a combination of self-reported intake with biomarkers may increase precision and help to better measure caffeine dose.

In summary, we show that although different measures of caffeine and caffeinated beverage intakes are highly correlated and have acceptable relative limits of agreement, absolute intakes differ significantly between measurement tools. These results highlight the importance of considering potential misclassification of caffeine exposure when assessing its effect on premenopausal women's health. Although we show that caffeinated beverage intake does not vary over the menstrual cycle, we did not assess differences in caffeine metabolism over the menstrual cycle. Further explorations examining the relationship between self-reported measures of caffeine and biomarkers of caffeine concentrations are needed.

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CHAPTER 5

CONCLUSION

Caffeine is the most widely consumed drug in the world and has been consumed through natural sources (primarily coffee and tea) for thousands of years, enjoyed for its ability to promote alertness and wakefulness (1, 2). Beverages with added caffeine, primarily sodas, are more recent arrivals but have quickly become a staple in many American diets (3).In addition to its stimulating effects, caffeine has been shown to reduce the risk of type 2 diabetes mellitus and obesity and while moderate consumption (≤ 400mg/day) has not been found to be associated with general toxicity, heart disease, osteoporosis, cancer or male fertility, reproductive-age women are considered to be 'at risk' and may require special advice in moderating intake (4-7). Several systematic reviews have not found a relationship between caffeine consumption and adverse reproductive outcomes (8, 9), but note that several methodological limitations, including exposure measurement error, limit the ability to rule out plausible alternative explanations (8).

The specific aims of our study were to 1) determine prospectively if *caffeine* or *caffeinated beverages* are related to serum concentrations of *reproductive hormones* and incident *anovulation*; 2) determine prospectively if *fructose* or *fructose-rich beverages* are related to serum concentrations of *reproductive hormones* and incident *anovulation*; and 3) assess the *validity* and *reproducibility* of the commonly used food frequency

questionnaire (FFQ) compared to the gold-standard of repeated 24-hour dietary recalls (24HDRs) among a healthy population of premenopausal women. Our goals for our study were to both help inform guidelines as to safe intakes of caffeinated beverages for women of reproductive age and improve methodology in how to best assess caffeine and caffeinated beverage exposure.

Key Findings

Regarding our first objective, we showed that caffeine intake was significantly associated with reproductive hormone levels and that this association varied across race/ethnicity groups. Higher caffeine and coffee intake was associated with decreased total and free estradiol (E2) concentrations among white women and increased total and free E2 concentrations among Asian women. In addition, caffeinated soda consumption was positively associated with increases in total and free E2 concentrations among all races. Caffeine consumption above the recommended levels was not associated with anovulation; however, any green tea consumption was associated with increased odds for anovulation. Though we observed differences by race, these results were based on a relatively small sample size and should be interpreted with caution. Additional research is needed to determine whether these relationships differ by race and mechanisms that might explain the differing effect.

Regarding our second objective, we showed that women who consumed more added sugar than an average American woman (≥ 73.2 grams/day) or above the 66th percentile in fructose intake (≥ 41.5 grams/day) had elevated free E2, follicular stimulating hormone (FSH) and luteinizing hormone (LH) concentrations compared to women who consumed less after adjusting for age, waist-to-hip ratio, race, dietary

factors, physical activity, and relevant hormones. No associations were found between intakes above the American Heart Association's recommended limits for added sugar intake (≥ 40 grams/day) and reproductive hormone levels across the cycle. Women who consumed ≥1 cup/day of sweetened soda had elevated free E2 (β=0.15 [95% CI: 0.06, 0.24]. Neither artificially sweetened soda intake nor fruit juice intake ≥1 cup/day was significantly associated with reproductive hormones. No associations were found between added sugars, fructose or beverage intake and anovulation. Even at moderate consumption levels, added sugars, total fructose, and sweetened soda were associated with elevated E2 concentrations among premenopausal women. Further research into whether fructose alone or in conjunction with other components in sweetened soda is associated with sex hormones is warranted.

Regarding our third objective, we demonstrated that caffeine intake reported in the 24HDRs was less than the FFQs despite high correlation and moderate agreement. Women also reported less caffeinated coffee intake in the 24HDR compared to their daily diary. Although caffeine and coffee exposures were highly correlated, absolute intakes differed significantly between measurement tools. We showed that while the FFQ may be appropriate for ranking caffeine and caffeinated beverage exposure, it may not be appropriate if the research aim is to classify exposure based on clinically relevant cutpoints. These results highlight the importance of considering potential misclassification of caffeine exposure and the need for further validation studies with biomarker assessment.

We additionally demonstrated that the FFQ is reproducible over 2 menstrual cycles (BioCycle Study period) and that baseline FFQ values reported for the previous 6-

month intake do not significantly differ from values recorded over the study period. Finally, repeated measures analyses of both the 8 24HDRs and daily diaries (captured over 2 cycles) indicated that neither caffeine nor caffeinated beverage intake varies within women over the course of the menstrual cycle. These results show that caffeine and caffeinated beverages are habitually consumed by women of reproductive age and methods that address episodically consumed foods are not necessary when evaluating caffeine or caffeinated beverage exposure. However, further studies assessing differences in caffeine metabolism over the menstrual cycle are still needed.

Public Health Implications

Our study revealed that inconsistent results regarding the effect of caffeine and caffeinated beverages on reproductive function may be due to in part to the inability of the FFQ to capture actual intakes. However, a biomarker validation study is needed to confirm these findings. Our study also revealed that moderate consumption of caffeine/fructose-rich beverages do not pose a risk for anovulatory infertility but may affect other chronic disease risk, such as reproductive cancers, via the small but chronic elevation or insufficiency of female reproductive hormones, particularly estrogen. Further research understanding both the direct and indirect effects of caffeine, fructose, and related beverages on reproductive health and how effects may differ by race and what other beverage components may be contributing to observed effects is needed.

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