TITLE OF THE MANUSCRIPT

Urinary concentrations of di(2-ethylhexyl) phthalate metabolites and serum reproductive hormones: Pooled analysis of fertile and infertile men

SHORT RUNNING TITLE

Phthalates and hormones in male population

AUTHORSHIP

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ABSTRACT

Urinary concentrations of metabolites of the anti-androgenic xenobiotic di-(2-ethylhexyl) phthalate (DEHP) were previously shown to be weakly associated with serum levels of several hormones in two disparate US populations; partners of pregnant women participating in the Study for Future Families, and partners in an infertile couple from Massachusetts General Hospital infertility clinic. The observed associations between phthalate metabolites and reproductive hormones were robust and insensitive to the characteristics of the subpopulation or the laboratory in which the hormones were measured, despite the fact that these two populations span a range of fertility, urinary phthalate metabolites and reproductive hormone levels. We therefore examined associations between urinary metabolites of DEHP and reproductive hormones (follicle stimulating hormone, luteinizing hormone, testosterone (T), inhibin B and estradiol (E_2), and sex hormone-binding globulin (SHGB) in the pooled population. The magnitude of the associations seen were similar to those reported for each population separately, but effect estimates were more precise due to the increased sample size, and the greater range of phthalate metabolite concentrations and hormone levels. Urinary concentrations of three metabolites of DEHP [mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)] were inversely associated with the free androgen index (FAI = T/SHBG) and calculated free testosterone (FT). Urinary concentrations of MEHHP and MEOHP were positively associated with SHBG, and MEHP was inversely associated with E_2. No other phthalate metabolites were associated with serum hormones, consistent with results in each population. Our results in this diverse population suggest that DEHP exposure is robustly associated with some male sex steroid hormones.

KEY WORDS: anti-androgens, DEHP metabolites, endocrine disruptor, male hormones

INTRODUCTION

Recent studies have reported secular shifts in male reproductive hormone levels (Andersson et al, 2007; Travison et al, 2007) which might be associated with decreases in semen quality (Carlsen et al, 1992; Swan et al, 2007). While exposure data are limited, it has been hypothesized that these changes may, at least in part, reflect the widespread use, and human exposure to, environmental endocrine-disrupting compounds (EDCs) (Jørgensen et al, 2010; Sharpe and Skakkebæk, 2008).

Phthalates, man-made chemicals extensively used in industry and commerce, are among the most widely studied EDCs, and several, including di(2-ethylhexyl) phthalate (DEHP) and di-n butyl phthalate (DBP) have been shown to have anti-androgenic activity (ATSDR, 2002; CDC, 2011). A growing body of literature has shown relationships between several of these phthalates and adverse reproduction and development (Hauser and Calafat, 2005; NRC, 1999; Talsness et al, 2009; Thompson et al, 2009). Laboratory studies have shown that DEHP and/or its metabolites are associated with the induction of testicular toxicity in neonatal, pubertal and adult rodents (Heindel et al, 1989; Li et al, 1998; 2000; Parmar et al, 1986; Srivastava et al, 1990). However, adult animals are usually less sensitive than young pubertal animals or animals exposed in utero (Dostal et al, 1988; Higuchi et al, 2003). For example, several toxicological studies have demonstrated that DEHP, DBP, benzylbutyl phthalate (BzBP), and di-isononyl phthalate (DiNP) disrupt reproductive tract development (e.g. hypospadias, reduced fetal testosterone synthesis) in male rodents due to anti-androgenic action (Gray et al, 2000; Parks et al, 2000).

Nevertheless, only a small number of human studies have investigated the relationship between male reproductive hormones and phthalate exposures. In those studies relationships have been shown between human prenatal and peri-natal exposure to some phthalate metabolites and alterations in reproductive hormones [sex hormone-binding

globulin (SHBG), luteinizing hormone (LH) and free testosterone (FT)] (Main et al, 2006), and markers of male reproductive development (Swan et al, 2005; Swan, 2008). In a population of young men, Jönsson et al. (2005) reported an inverse association between urinary monoethyl phthalate (MEP) concentrations and circulating LH, though no associations were found between other phthalate metabolites and reproductive hormones. Pan et al. (2006) studied adult men occupationally exposed to some phthalates (DEHP and DBP), and reported that phthalate exposure was inversely associated with serum FT levels. Meeker and collaborators (2009) investigated this issue and extended their previous work (Duty et al, 2005) by including a larger sample size and expanding the number of hormones and phthalate metabolites measured. In a male population attending a fertility clinic, the authors reported an association between increased urinary concentration of mono(2-ethylhexyl) phthalate (MEHP) with decreased testosterone (T), estradiol (E_2) and free androgen index (FAI) levels, showing that exposure to DEHP might be associated with altered steroid hormones in these men. Recently, Mendiola et al. (2010) investigated these associations in a population of fertile men. Both Meeker et al. (2009) and Mendiola et al. (2010) showed significant inverse association between FAI levels and urinary concentrations of several DEHP metabolites. In both studies SHBG was positively associated with urinary concentrations of MEHP, but not with other DEHP metabolites. Neither study found notable associations between metabolites of any other phthalate and hormones under investigation. There were, however, some discrepancies between these studies. For instance, Duty et al. (2005) reported a dose-response relationship between monobenzyl phthalate (MBzP) and follicle stimulating hormone (FSH) and mono-n-butyl phthalate (MBP) and inhibin B but no strong evidence of an association between MEHP and T. Meeker et al. (2009) reported a significant relationship between MEHP and T, and mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethyl-5-oxohexyl)

phthalate (MEOHP) and FAI ($p<.05$) but for FAI and MEHP the adjusted p-value was <0.1. Mendiola et al. (2010) reported a significant association between several DEHP metabolites and FAI but no relationship between DEHP metabolites and T.

The aim of the current study was to use a pooled analysis of a large heterogeneous population of both fertile (Mendiola et al, 2010) and infertile men (Meeker et al, 2009) to more precisely examine the relationships of urinary phthalate metabolite concentrations with serum reproductive hormone levels. Although data from both populations were previously published, this new pooled analysis adds to our understanding of the human health effects of phthalates by allowing us to systematically investigate whether associations differed by populations based on fertility status.

MATERIALS AND METHODS

Study populations

The present study includes men from two large ongoing studies of environmental influences on reproductive health. One of these, the Study for Future Families (SFF) (n=425), is a multicenter study of pregnant women and their male partners, conducted at prenatal clinics affiliated with university hospitals in five US cities (Harbor-UCLA and Cedars-Sinai Medical Center in Los Angeles, CA; University of Minnesota Medical Center in Minneapolis, MN; University Physicians in Columbia, MO; Mt. Sinai School of Medicine, New York City, NY and University of Iowa, Iowa City, IA) between 1999 and 2005. In this study couples were eligible only if the pregnancy was conceived without assisted reproduction (Swan et al, 2003). The second study included men who were male partners of infertile couples seeking evaluation at the Vincent Memorial Obstetrics and Gynecology Service, Andrology Laboratory and In Vitro Fertilization Unit, Massachusetts General Hospital (MGH) (n=425) in Boston between January 2000 and May 2004 (Meeker

et al, 2009). That infertility clinic population includes men with male factor infertility as well as men who are partners of women with female factor infertility. Methods for clinical examination, data collection, and semen analysis have been described previously for each study (Meeker et al, 2009; Swan et al, 2003). Briefly, in both studies the men completed a questionnaire and gave urine, blood and semen specimens. Information was collected on demographics, medical history, and lifestyle factors. Human subject approvals were obtained from Institutional Review Boards at all participating institutions. The involvement of Centers for Disease Control and Prevention (CDC) laboratory in SFF was limited and determined not to constitute engagement in human subjects research.

Serum hormone analysis

In both populations venous blood samples were drawn, and the serum was separated and frozen at – 80ºC, on the same day the urinary sample was collected. Samples were analyzed for hormones in two different laboratories, SFF samples at the Rigshospitalet Andrology Laboratory (Copenhagen, Denmark) and MGH samples at the REU Laboratory at MGH, Boston, MA. Each methodology has been described previously elsewhere (Asklund et al, 2007; Bang et al, 2005; Meeker et al, 2009; Mendiola et al, 2010). The MGH lab is a Clinical Laboratory Improvement Amendments (CLIA)-certified (Centers for Medicare and Medicaid Services, Department of Health and Human Services, Baltimore, MD, USA) and the Rigshospitalet Andrology Laboratory participates in Bio-Rad Laboratories external quality Immunoassay program (Bio-Rad Laboratories, Copenhagen, Denmark). Table 1 summarizes the serum hormone analysis methods that were employed at the two laboratories. FAI was calculated as total testosterone ×100/ SHBG, and FT concentration was calculated using the equation of Vermeulen et al. (1999).

Urinary phthalate metabolites measures

In both populations the concentrations of urinary phthalate metabolites were determined at the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA), which had no access to participant data. SFF samples were analyzed in 2006 and MGH samples were analyzed throughout a 3-year period (2003-2006). Urinary samples were frozen and stored at -80 ºC, and then shipped to CDC on dry ice. Phthalate metabolites were measured in urine to avoid potential sample contamination from the parent diester and because the metabolites (not the parent diesters) are the active toxicants (Li et al, 1998). The analytical approach for the analysis of urinary phthalate metabolites in the MGH men population has been previously described (Meeker et al, 2009; Silva et al, 2007). A modification of that approach was used in the SFF population and has been described and published elsewhere (Swan et al, 2005). Limits of detection (LOD) are in the low nanogram per milliliter range (see Table 4). Isotopically labeled internal standards were used along with conjugated internal standards to increase precision and accuracy of the measurements. The method is accurate (spiked recoveries are near 100%), and precise with between-day relative standard deviations of < 10%. Quality control samples and laboratory blanks were analyzed along with unknown samples to monitor performance of the method (Swan et al, 2005). Concentrations are reported in ng/mL. While different metabolites were assessed in our separate studies, we report here only the six urinary phthalate metabolites that were measured in both populations: MEHP, MEHHP, MEOHP, MEP, MBzP and MBP (as sum of MBP and mono-iso-butyl phthalate concentrations). We also calculated the percent of these DEHP metabolites excreted as MEHP (MEHP%). To calculate MEHP%, we converted MEHP, MEHHP and MEOHP concentrations to nanomoles per milliliter, divided MEHP

200 concentrations by the sum of concentrations of MEOHP, MEHHP and MEHP, and
201 multiplied by 100 (Hauser et al, 2006).
202
203 **Statistical analyses**
204 Data from Meeker et al. (2009) and Mendiola et al. (2010) were pooled for statistical
205 analysis. Serum hormones (except E_2) and urinary phthalate metabolite concentrations
206 were log transformed ($\log_{10}$) to normalize their asymmetric distributions. In preliminary
207 analyses, we used Mann-Whitney U test and Pearson correlation coefficients to explore the
208 relationship between each hormone concentration and each phthalate metabolite
209 concentration. We then used multiple linear regression analysis to control for appropriate
210 covariates, including age, age square, body mass index (BMI), smoking status (current
211 smoker vs. never smoked), ethnicity (African American vs. others), time of sample
212 collection (hours after 7:00 am), and time of sample collection squared. Urinary dilution
213 was measured differently in the two populations; SFF models were adjusted by urinary
214 creatinine concentrations and MGH models by specific gravity (SG). Although these
215 methods of adjusting for urinary concentration are different, the rank of urinary
216 concentrations assigned by each method should be comparable (Box and Tidwell 1962).
217 Therefore, the measure of urinary dilution used in the combined analysis was the rank of
218 creatinine or SG in the respective data sets. We also included a term for study center (SFF
219 vs. MGH), which reflects between-center differences, including those due to differing
220 methods of hormone analysis and measurement for urinary dilution. Age, BMI and time of
221 collection were modeled as continuous variables, all others as dichotomous indicator
222 variables. Most metabolite concentrations were above the LOD; those below the LOD were
223 assigned the value LOD divided by the square root of 2, which has been recommended
224 when the data are not highly skewed (i.e. geometric standard deviation <3) (Hornung and

225 Reed 1990), as was the case in the present analysis. Two analysts (J.D.M. and J.M.)
226 conducted all analyses independently using SAS version 9.1 (SAS Institute Inc., Cary, NC,
227 USA) and SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).
228
229 **RESULTS**
230 Serendipitously, 425 men in each population provided urine and blood. Estradiol and
231 inhibin B serum levels were available for 830 and 849 males respectively and 783 had
232 complete information on all covariates and were included in the final multivariate analyses.
233 MEHHP and MEOHP urinary concentrations were measured in 646 men, as these
234 metabolites were not incorporated into the MGH study until after the study had already
235 begun. Basic demographic data are presented in Table 2, including information about
236 reproductive parameters in the separate and joint populations; Figures 1a-1g present the
237 frequency distribution of the reproductive hormones measured in the two populations.
238 Summary statistics for the serum concentrations of men's reproductive hormones are
239 presented in Table 3. All hormone levels differed significantly between the two
240 populations. Both FSH and LH were about three-fold higher in MGH men compared to
241 SFF men, and inhibin B levels were lower in MGH men.
242 The urinary concentrations (in ng/mL) of DEHP metabolites (before urine dilution
243 adjustment) are shown in Table 4, together with the LOD and percent of samples above the
244 LOD. Urinary concentrations of DEHP metabolites were notably higher in MGH men than
245 men in SFF, while MEP, MBP and MBzP were higher in SFF men. MEHP% was similar
246 in the two populations.
247 Table 5 shows correlation coefficients for reproductive hormones and unadjusted urinary
248 DEHP metabolite concentrations from initial bivariate analyses. We observed no
249 associations between any hormone levels and any urinary metabolites of phthalates other

250 than DEHP (data available on request). Therefore, here we report only the associations
251 involving the three measured metabolites of DEHP (MEHP, MEHHP and MEOHP).
252 Table 6 shows the results of the multivariate analysis for reproductive hormones and
253 urinary DEHP metabolite concentrations in both populations separately and combined.
254 After adjustment for covariates many of the relationships (as described by the β
255 coefficients) were consistent with previously published results (Meeker et al, 2009;
256 Mendiola et al, 2010), though the effect estimate for E_2 strengthened in the pooled
257 analysis. Overall, an increase in statistical power due to increased sample size resulted in
258 increased precision in the effect estimates compared to the individual studies. There were
259 no significant associations between T and any urinary DEHP metabolites. FAI and FT
260 were both inversely associated with the urinary concentrations of all three urinary DEHP
261 metabolites measured in the study (MEHP, MEHHP and MEOHP). Serum gonadotropin
262 levels (FSH and LH) were not associated with DEHP metabolite concentrations in the
263 separate or combined populations. There was a significant inverse association between E_2
264 levels and urinary MEHP concentrations, but not with the other DEHP metabolites. T/E_2
265 ratio was positively associated with urinary MEHP metabolite concentrations. SHBG
266 levels were positively related to urinary MEHHP and MEOHP concentrations but not
267 MEHP concentration. Figure 2 shows the percent change in men's reproductive hormones
268 expected with an inter-quartile increase in urinary DEHP metabolite concentrations for a
269 34-year-old non-smoker with BMI of 28 kg/m^2. For this typical subject, an increase in
270 urinary concentrations of MEHP and the oxidative metabolites (MEHHP and MEOHP)
271 from the 25^{th} to the 75^{th} percentile would be predicted to decrease steroid hormone levels
272 the amount ranging from 3.5% and 7%, for T and E_2 respectively.
273
274

DISCUSSION

This is the first study to examine the associations between urinary concentrations of phthalate metabolites and reproductive hormone serum levels in a large cohort including both fertile men and male partners of infertile couples. Our results suggest that exposure to DEHP at environmental concentrations is associated with statistically significant declines in free testosterone (both FAI and FT) and serum estradiol (E_2). The other phthalate monoester metabolites we examined (MEP, MBP and MBzP) were not associated with any reproductive hormones. These associations are not substantially different from those reported in the separate analyses, which in turn do not differ appreciably between the two populations (Meeker et al, 2009; Mendiola et al, 2010). However, each of the individual studies provides information only about a limited subset of the total population. When the two populations are combined, the effect estimates are more precise and more generalizable to men of reproductive age.

In this combined population of fertile and subfertile men, we saw no significant associations with total T levels and any phthalate metabolites. However, both FT and FAI were both inversely associated with urinary DEHP metabolite concentrations. This may be accounted for by a positive association between serum SHBG levels and urinary MEHP concentrations in the SFF cohort and with MEHHP and MEOHP in the combined analysis. Significant positive associations were seen between SHBG and MEHHP and MEOHP in the combined analysis. However, associations between SHBG and MEHP differed in these two cohorts, with a significant positive association in SFF men, but a non-significant negative association in the MGH cohort. This resulted in a non-significant positive association between SHBG and MEHP in the combined analyses. It should be noted that the serum SHBG concentration in all the subjects are within the physiological range of

299 adult men. Thus, the small increases in serum SHBG levels associated with greater DEHP
300 may result in a small reduction of FT without affecting the total serum T levels.
301 We did not see an association between DEHP metabolite concentrations and LH in this
302 combined population of fertile and infertile men. In this mixed population the small
303 changes in FT and FAI associated with DEHP may not be sufficient to elicit the negative
304 feedback that would be expected to produce a positive association between LH and DEHP
305 metabolites.
306 Although all men had serum steroid hormones within the laboratory reference ranges, our
307 findings suggest a somewhat anti-androgenic effect of DEHP. This is consistent with data
308 showing that phthalates may inhibit expression of genes involved in steroidogenesis
309 (cholesterol transport and the biosynthesis of testosterone) in rat fetal testis after in-utero
310 exposure to large doses of DEHP (Borch et al, 2006).
311 Estradiol plays a role in male germ cell survival in vitro (Pentikainen et al, 2000). In our
312 study urinary MEHP concentrations were inversely associated with serum E_2 levels and
313 positively associated with T/E_2 ratio. In vitro and animal studies have shown that
314 aromatase activity, and E_2 production, can be lowered by DEHP and/or MEHP (Andrade et
315 al, 2006; Davis et al, 1994; Lovekamp and Davis, 2001; Noda et al, 2007). Our results
316 suggest that, as in rodent models, DEHP may be associated with a reduced aromatase
317 activity.
318 We compared unadjusted urinary concentrations of DEHP metabolites in our subjects to
319 those from men participants in the 2007-2008 National Health and Nutrition Examination
320 Survey (NHANES) (CDC, 2011). Median MEHP concentration was almost twice as high
321 in our combined population (4.4 ng/mL compared to 2.3 ng/mL), while the other DEHP
322 metabolites were similar (e.g., medians 20.9 and 23.2 ng/mL for MEHHP in NHANES and
323 our population).

Our data were limited by the use of a single urine and blood sample to assess DEHP exposure and hormone function, respectively. However, several studies have reported that although phthalate metabolite concentrations are variable within an individual over time, the average concentration over the course of days, weeks or months can be satisfactorily predicted by a single sample (Hauser et al, 2004; Hoppin et al, 2002; Teitelbaum et al, 2008). Similarly, a single sample can be used to classify reproductive hormone levels in men (Bjornerem et al, 2006).

It is generally accepted that hormone levels obtained in different laboratories or/and with different methods are likely to differ. The variations among laboratories are more marked for steroid hormone levels at low levels (e.g. T and E_2 levels in men) than for gonadotropins (Pitteloud et al, 2008; Rosner et al, 2007; Sikaris et al, 2005; Taieb et al, 2003; Wang et al, 2004). We included a center effect in our multivariate models to reflect between-laboratory differences. Adding this covariate did not alter associations between urinary DEHP metabolites and androgens (T, FT and FAI). However, it did slightly increase effect estimates for E_2 and SHBG and decreased them for LH and FSH.

One limitation of all previously published studies on phthalate metabolites and reproductive parameters is that their study populations (fertile men or men in infertility clinics) are not representative of the general population. Our combined analysis includes a wider range of men, though still not a representative sample of adult men.

In conclusion, our results in this population, including both fertile and infertile men, suggest that DEHP exposure is associated with some changes in circulating levels of male sex steroid hormones, consistent with the known anti-androgenic effect of this chemical.

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FIGURE LEGENDS

Figures 1a-1g. Distribution (density) of the reproductive hormone profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

Figure 2. Percent change in men's reproductive hormones expected with an increase from the 25^{th} to the 75^{th} percentile in DEHP metabolite concentrations for a standard subject (34 years old, non-smoker with BMI of 28 kg/m2). Error bars indicate the 95% confidence intervals.

Table 1. Methods for serum hormone analyses at the two laboratories (MGH and SFF).

MGH assay details					
Hormone	Method	Manufacturer/System	Sensitivity	CVs	
				Intra-assay	Inter-assay
FSH	Microparticle Enzyme Immunoassay (MEIA)	Abbott AxSYM	1.1 IU/L	3-7%	2-5%
LH	Microparticle Enzyme Immunoassay (MEIA)	Abbott AxSYM	1.2 IU/L	4-7%	2-7%
Testosterone	Radioimmunoassay (RIA)	Coat-A-Count kit, Diagnostic Products Corp.	0.14 nmol/L	10%	12%
SHBG	Solid-phase two-site enzyme chemiluminescent immunometric assay	Immulite, Diagnostic Products Corp.	1 nmol/L	2-5%	4-8%
Inhibin B	Double antibody enzyme-linked immunosorbent assay (Double antibody ELISA)	Oxford Bioinnovation	50 pg/mL	8%	20%
Estradiol	Microparticle Enzyme Immunoassay (MEIA)	Abbott AxSYM	73 pmol/L	3-11%	5-15%

SFF assay details					
Hormone	Method	Manufacturer/System	Sensitivity	CVs	
				Intra-assay	Inter-assay
FSH	Time-resolved immunofluorometric assay (TR-IFMA)	DELFIA, Perkin Elmer	0.05 IU/L	1.3-2.1 %	2.8-4.6 %
LH	Time-resolved immunofluorometric assay (TR-IFMA)	DELFIA, Perkin Elmer	0.05 IU/L	1.5-3.0 %	4.0-4.5 %
Testosterone	Time-resolved fluoroimmunoassay (TR-FIA)	DELFIA, Perkin Elmer	0.23 nmol/L	1.4-2 %	6-8 %
SHBG	Time-resolved immunofluorometric assay (TR-IFMA)	DELFIA, Perkin Elmer	0.23 nmol/L	3-5 %	4-5 %
Inhibin B	Specific two-sided enzyme immunometric assay	(Oxford Bioinnovation, in-house standard)	20 pg/ml	15 %	18 %
Estradiol	Radioimmunoassay (RIA)	Pantex, USA	18 pmol/L	3-8 %	11-13 %

CVs: coefficients of variation

Table 2. Characteristics of the SFF and MGH study populations

	SFF N=425	MGH N=425	Total N=850
	Mean (SD)		
Age (years)	32.2 (6.2)	36 (5.3)	34.3 (6.1)
BMI (kg/m^2)	28.2 (5.4)	28 (4.5)	28.1 (4.9)
	Percent of men		
Current smoker	21	9	15
White, non Hispanic	72.3	85	79
Sperm concentration < 20 $x10^6$/mL	7.8	15.3	12
Sperm motility (A+B) < 50%	37.4	45.9	42
Made a partner pregnant[a]	100	41.6	71
Had trouble fathering a child[b]	4.3	100	52

SFF: Study for Future Families
MGH: Massachusetts General Hospital
SD: Standard deviation

[a] In SFF, all men were partners of pregnant women. In MGH, this is the percent of men who self-reported that they had 'ever made a partner pregnant'

[b] In MGH, all men were in a couple seeking evaluation or treatment for infertility. In SFF this is the percent of men who responded positively to the question: 'Have you ever seen a doctor because you thought you might be having trouble fathering a child?'

Table 3. Summary statistics for serum reproductive hormone levels in men from both studies separately and combined (N=850)

	Study	Geometric Mean	Selected Percentiles			P value[e]
			10th	50th	90th	
Variables						
FSH (IU/L)						
	SFF	2.9	1.5	2.9	5.5	
	MGH	8.0	4.3	7.5	15.7	
	TOTAL	4.8	1.9	4.8	11.8	< 0.001
LH (IU/L)						
	SFF	3.3	1.9	3.3	5.4	
	MGH	10.1	5.8	9.9	17.2	
	TOTAL	5.7	2.4	5.5	14.5	< 0.001
T (nmol/L)						
	SFF	17.7	10.9	18.1	28.7	
	MGH	13.8	8.8	14.1	21.1	
	TOTAL	15.6	9.7	15.9	25	< 0.001
Inhibin B (pg/mL)						
	SFF[a]	207	120	218	333	
	MGH	147	81.6	160	262	
	TOTAL[c]	174	101	182	299	< 0.001
E_2 (pmol/L)						
	SFF[b]	80.1	53	83	121	
	MGH	96.2	36.7	110	165	
	TOTAL[d]	88	36.8	94.5	143	< 0.001
SHBG (nmol/L)						
	SFF	27.6	15	28	50.4	
	MGH	25.8	15.3	26	44	
	TOTAL	26.7	15	27	47	0.01
FAI						
	SFF	64.2	39.7	65	100	
	MGH	53.4	34.9	52.1	83.6	
	TOTAL	58.6	37.1	58.4	93.8	< 0.001
FT						
	SFF	11.5	7.4	11.8	17.5	
	MGH	9.0	6.1	9.1	12.9	
	TOTAL	10.2	6.5	10.3	15.9	< 0.001
T/E_2 ratio						
	SFF[b]	0.22	0.11	0.23	0.40	
	MGH	0.14	0.08	0.13	0.32	
	TOTAL[d]	0.18	0.09	0.18	0.37	< 0.001

[a]N= 424 for Inhibin B
[b]N= 405 for E_2 and T/E_2 ratio
[c]N= 849 for Inhibin B
[d]N= 830 for E_2 and T/E_2 ratio, [e]Mann-Whitney U Test

Table 4. Summary statistics for the urinary concentrations (in ng/mL) of DEHP metabolites (non creatinine-adjusted) in men from both studies separately and combined (N=850)

	Study	Geometric Mean			Selected Percentiles			P value[f]
			LOD[a]	% > LOD[b]	10th	50th	90th	
Variables								
MEHP (ng/mL)								
	SFF	3.7	1.2	77	0.85	3.2	17.8	
	MGH	8.2	1.0	83	1.0	7.9	64.3	
	TOTAL	4.9		80	0.9	4.4	39.2	< 0.001
MEHHP (ng/mL)								
	SFF	23.3	0.7	99	4.6	23.7	104	
	MGH[c]	55.6	1.3	100	13.2	47.0	272	
	TOTAL[d]	27.6		99.5	5.4	25.3	170	< 0.001
MEOHP (ng/mL)								
	SFF	12.9	0.7	97	2.7	12.9	57.4	
	MGH[c]	36.2	1.1	99	8.4	32.2	193	
	TOTAL[d]	16.1		98	3.2	15.4	110	< 0.001
MEP (ng/mL)								
	SFF	206	0.8	100	31.8	205	1358	
	MGH	179	1.1	100	30.2	153	1376	
	TOTAL	173		100	23.6	170	1259	< 0.001
MBP (ng/mL)								
	SFF	19.2	0.6	98	4.0	24.5	65.3	
	MGH	17.1	0.8	97	5.1	17.7	50.8	
	TOTAL	16.3		97.5	3.4	18.8	58.2	< 0.001
MBzP (ng/mL)								
	SFF	11.2	0.3	98	2.1	12.5	49.8	
	MGH	7.7	0.7	97	2.3	8.2	24.9	
	TOTAL	8.4		97.5	1.6	9.8	41.2	< 0.001
MEHP%[e]								
	SFF	9.4			3.9	10.1	18.8	
	MGH[c]	9.4			3.5	10.3	24.3	
	TOTAL[d]	9.4			3.7	10.1	21.6	0.59

[a]Limit of detection (LOD) in ng/mL for each phthalate metabolite.
[b]Percentage of samples above the LOD for each phthalate metabolite
[c]N= 221
[d]N= 646
[e]To calculate MEHP%, we transformed MEHP, MEHHP and MEOHP concentrations to nanomoles per milliliter, divided MEHP levels by the sum of concentrations of MEHP, MEHHP and MEOHP, and then multiplied by 100
[f]Mann-Whitney U Test

Table 5. Correlation coefficients for reproductive hormones and DEHP metabolites[1] concentrations in men (bivariate analysis) (n=850)

	Study	MEHP		MEHHP		MEOHP	
		R	95% CI	R	95% CI	R	95% CI
FSH							
	SFF	.003	(-.09, .10)	-.01	(-.11, .09)	-.01	(-.11, .09)
	MGH	.04	(-.05, .14)	-.03	(-.16, .10)	-.04	(-.17, .09)
	TOTAL	.16	(.09, .23)[d]	.10	(.03, .18)[d]	.14	(.06, .22)[d]
LH							
	SFF	-.01	(-.11, .09)	-.02	(-.12, .08)	-.03	(-.13, .07)
	MGH	-.04	(-.14, .05)	-.01	(-.15, .12)	-.01	(-.14, .13)
	TOTAL	.14	(.07, .21)[d]	.12	(.04, .20)[d]	.16	(.08, .24)[d]
T							
	SFF	-.07	(-.17, .03)	-.09	(-.19, .01)	-.10	(-.20, -.001)[c]
	MGH	-.15	(-.24, -.05)[c]	-.13	(-.25, .001)	-.12	(-.25, .01)
	TOTAL	-.16	(-.23,-.10)[d]	-.15	(-.23, -.08)[d]	-.17	(-.25, -.09)[d]
E_2							
	SFF[a]	-.06	(-.16, .04)	-.02	(-.12, .08)	-.02	(-.12, .08)
	MGH	-.12	(-.22, -.03)[c]	-.07	(-.20, .06)	-.06	(-.19, .08)
	TOTAL[b]	-.04	(-.10, .03)	-.02	(-.10, .06)	-.01	(-.09, .07)
SHBG							
	SFF	.06	(-.04, .16)	-.03	(-.13, .07)	-.03	(-.13, .07)
	MGH	-.05	(-.15, .05)	.03	(-.10, .16)	.04	(-.09, .17)
	TOTAL	-.01	(-.08, .05)	-.02	(-.10, .06)	-.02	(-.10, .06)
FAI							
	SFF	-.15	(-.25, -.06)[d]	-.06	(-.16, .04)	-.07	(-.17, .03)
	MGH	-.08	(-.18, .01)	-.17	(-.29, -.04)[d]	-.17	(-.29, -.04)[d]
	TOTAL	-.15	(-.22,-.09)[d]	-.14	(-.21, -.06)[d]	-.16	(-.23, -.08)[d]
FT							
	SFF	-.12	(-.22, -.03)[d]	-.09	(-.19, .01)	-.10	(-.20, -.001)[c]
	MGH	-.16	(-.25, -.06)[d]	-.19	(-.31, -.06)[d]	-.19	(-.31, -.05)[d]
	TOTAL	-.19	(-.26,-.13)[d]	-.17	(-.25, -.10)[d]	-.19	(-.27, -.12)[d]
T/E_2							
	SFF[a]	-.002	(-.10, .10)	-.05	(-.15, .05)	-.06	(-.16, .04)
	MGH	.03	(-.07, .13)	-.01	(-.14, .12)	-.02	(-.15, .11)
	TOTAL[b]	-.07	(-.14, .-.01)[c]	-.09	(-.17, -.01)[c]	-.11	(-.19, -.03)[d]

[1] non-creatinine-adjusted/non-SG-adjusted
[a]N= 405 for E_2
[b]N= 830 for E_2
[c]P value≤.05
[d]P value≤.01
R= correlation coefficient
CI= confidence interval
Log-transformations of phthalate metabolites and men sex hormones, except for E_2 were used

Table 6. Multivariate analysis for reproductive hormones and DEHP metabolites concentrations in men (n=783)[1]

	Study	MEHP		MEHHP		MEOHP	
		β	95% CI	β	95% CI	β	95% CI
FSH							
	SFF	.01	(-.03, .06)	.01	(-.04, .06)	.01	(-.04, .05)
	MGH	.02	(-.02, .05)	-.02	(-.07, .04)	-.02	(-.07, .03)
	TOTAL	.01	(-.01, .04)	-.01	(-.04, .03)	-.01	(-.05, .02)
LH							
	SFF	.01	(-.03, .05)	-.01	(-.05, .03)	-.01	(-.05, .03)
	MGH	-.01	(-.04, .02)	-.002	(-.05, .04)	.001	(-.04, .05)
	TOTAL	-.01	(-.03, .01)	-.02	(-.05, .01)	-.02	(-.05, .01)
T							
	SFF	.01	(-.03, .04)	-.01	(-.04, .03)	-.01	(-.04, .03)
	MGH	-.02	(-.04, -.003)[c]	-.02	(-.05, .01)	-.02	(-.05, .02)
	TOTAL	-.01	(-.03, .005)	-.01	(-.03, .02)	-.01	(-.03, .02)
E_2							
	SFF[a]	-4.6	(-10.4, 1.1)	-2.6	(-8.4, 3.2)	-2.9	(-8.8, 3.0)
	MGH	-3.1	(-5.7, -.46)[c]	-1.9	(-5.9, 2.1)	-1.5	(-5.3, 2.4)
	TOTAL[b]	-7.9	(-12.4, -3.5)[d]	-3.4	(-8.8, 1.9)	-3.0	(-8.4, 2.3)
SHBG							
	SFF	.05	(.02, .09)[d]	.03	(-.01, .07)	.03	(-.01, .07)
	MGH	-.01	(-.03, .02)	.02	(-.02, .06)	.03	(-.01, .07)
	TOTAL	.01	(-.01, .03)	.03	(.002, .06)[c]	.03	(.005, .06)[c]
FAI							
	SFF	-.05	(-.08, -.02)[d]	-.04	(-.07, -.004)[d]	-.04	(-.07, -.01)[d]
	MGH	-.01	(-.03, .01)	-.03	(-.06, -.001)[c]	-.03	(-.06, -.002)[c]
	TOTAL	-.02	(-.04, -.01)[c]	-.04	(-.06, -.01)[d]	-.04	(-.06, -.01)[d]
FT							
	SFF	-.02	(-.05, .01)	-.02	(-.05, .01)	-.02	(-.05, .01)
	MGH	-.02	(-.04, -.004)[c]	-.03	(-.06, .001)	-.03	(-.06, .001)
	TOTAL	-.02	(-.04, -.004)[c]	-.02	(-.04, -.001)[c]	-.02	(-.04, -.001)[c]
T/E_2							
	SFF[a]	.03	(-.02, .07)	.004	(-.04, .05)	.003	(-.04, .05)
	MGH	.03	(-.008, .06)	.02	(-.04, .08)	.02	(-.04, .08)
	TOTAL[b]	.03	(.001, .05)[c]	.01	(-.02, .05)	.01	(-.03, .05)

[1]Controlling for age, age square, BMI, smoking status (current smoker vs. never smoked), ethnicity (African-American vs. others), study center (SFF vs. MGH), time of sample collection and time of sample collection squared. In addition, to take into account urinary dilution, SFF model also was adjusted by urinary creatinine values, MGH by specific gravity and the joint analysis (TOTAL) by ranking both variables.

[a]N= 346 for E_2

[b]N= 766 for E_2

[c]P value≤.05, [d]P value≤.01

β= regression coefficient, CI= confidence interval

Log-transformations of phthalate metabolites and men sex hormones, except for E_2 were used

Figure 1a. Distribution (density) of the serum follicle stimulating hormone (FSH) profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

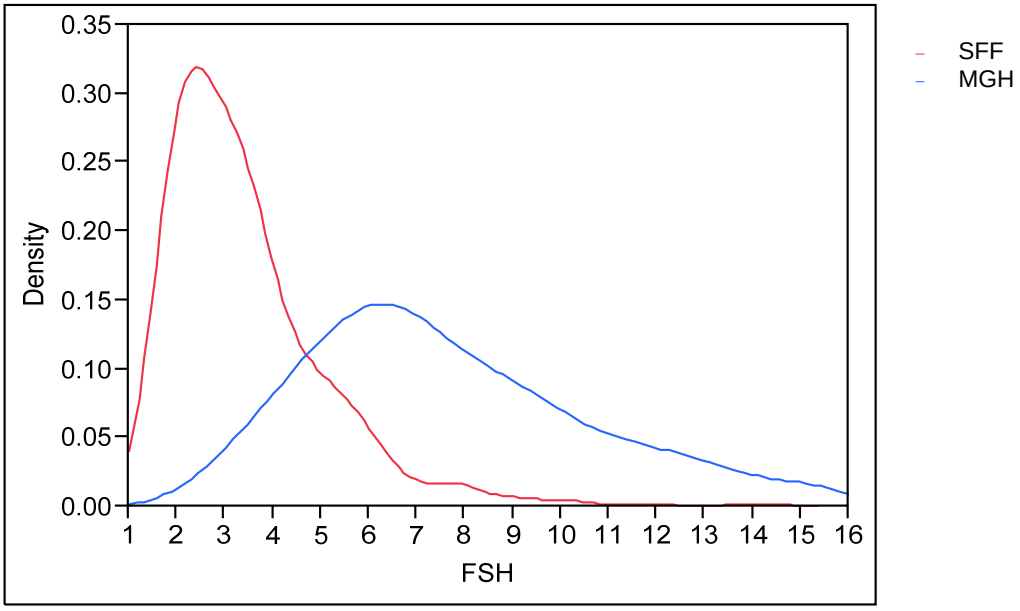

Figure 1b. Distribution (density) of the serum luteinizing hormone (LH) profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

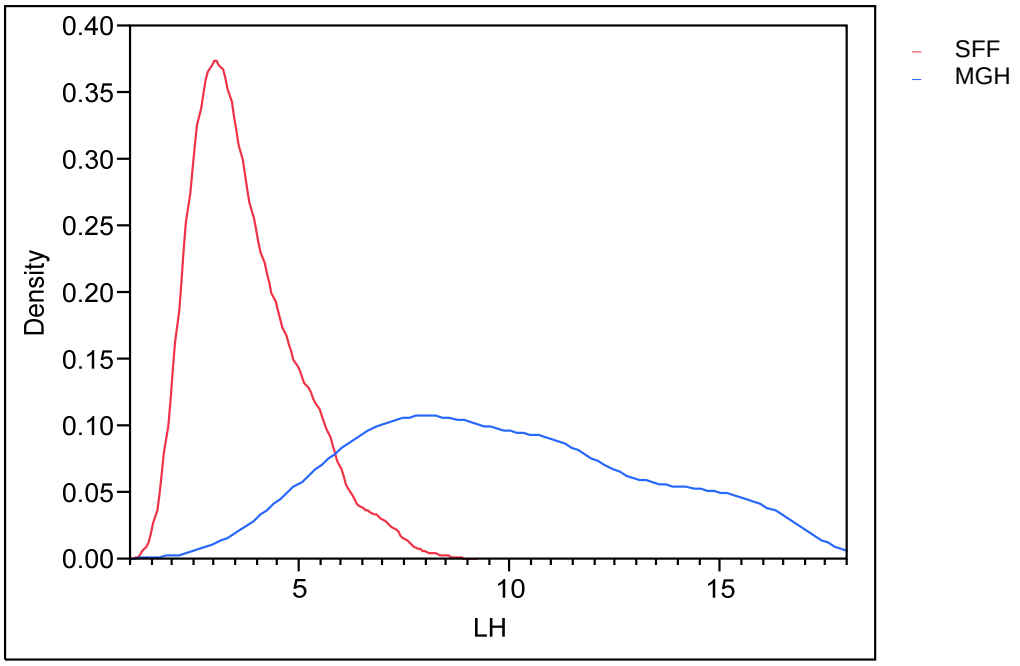

Figure 1c. Distribution (density) of the serum inhibin B profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

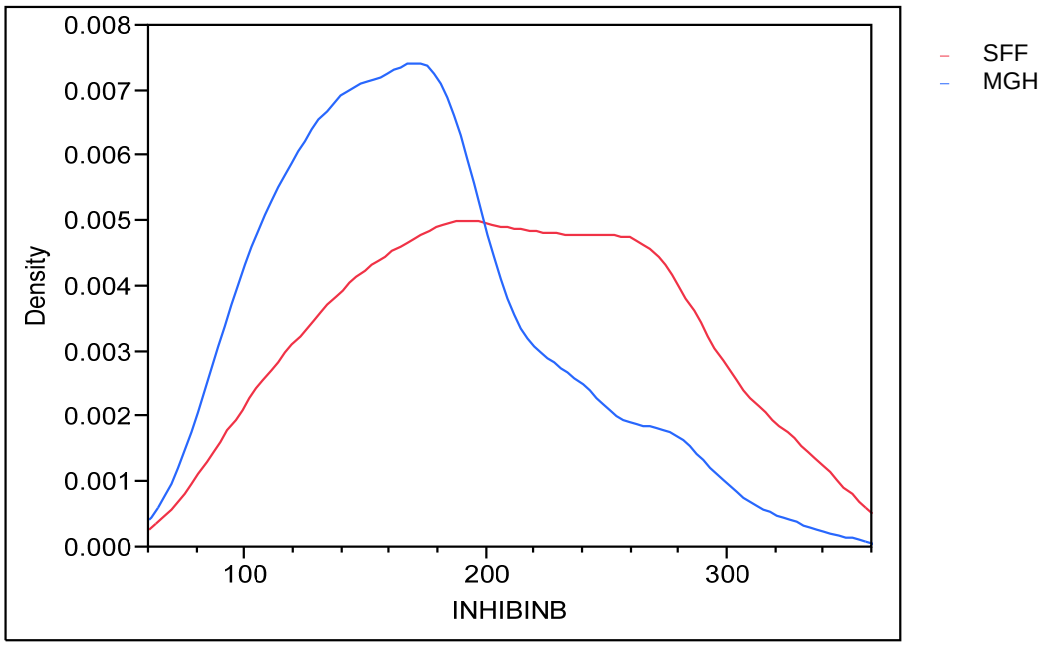

Figure 1d. Distribution (density) of the serum estradiol (E_2) profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

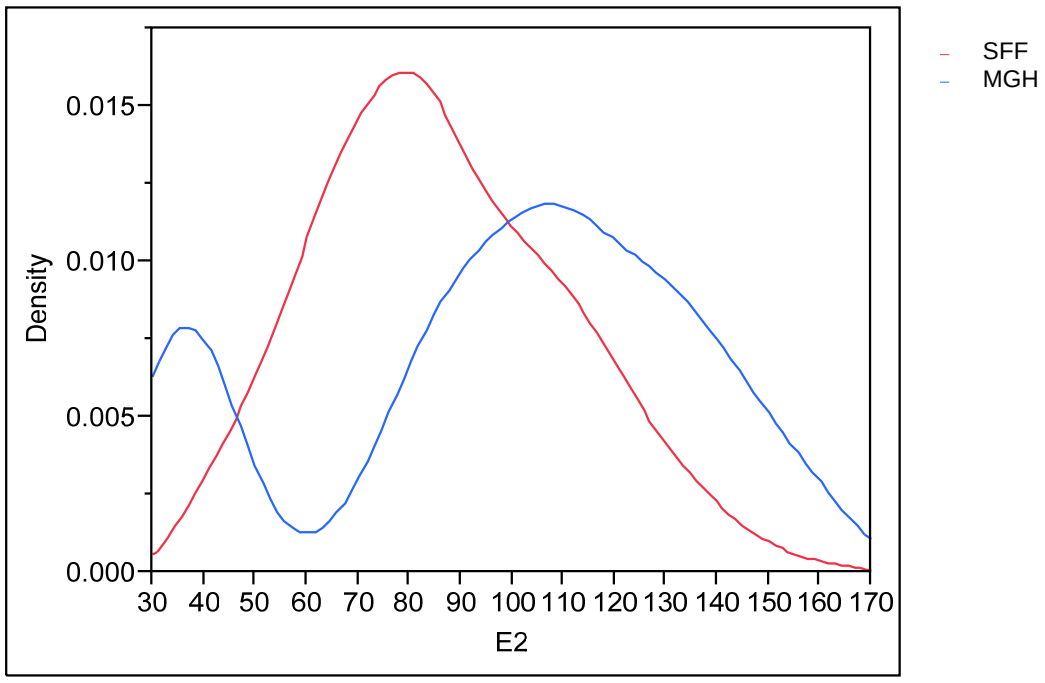

Figure 1e. Distribution (density) of the free androgen index (FAI) profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

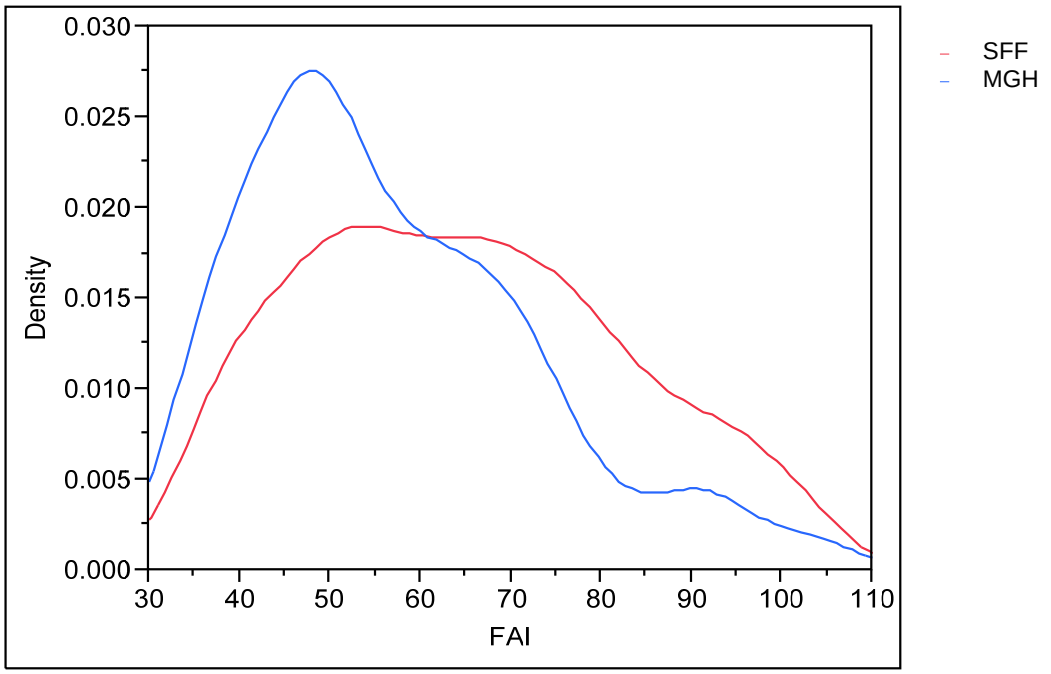

Figure 1f. Distribution (density) of the free testosterone (FT) profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

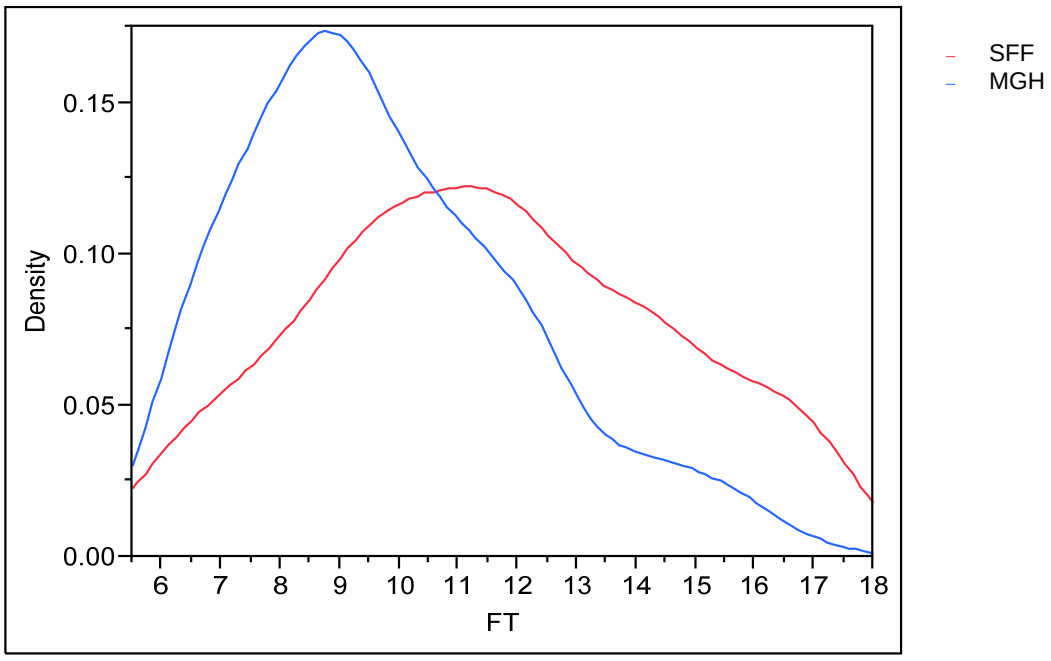

Figure 1g. Distribution (density) of the serum testosterone (T) profiles for SFF and MHG. All data have been truncated to fall between the 5th and 95th percentiles.

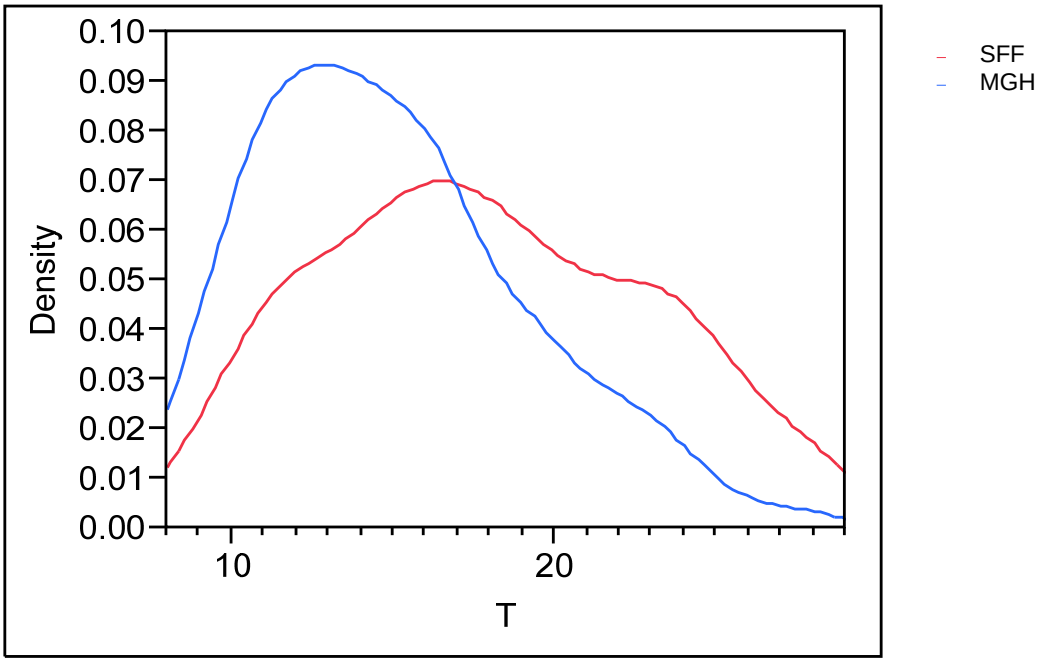

Figure 2. Percent change in men's reproductive hormones expected with an increase from the 25th to the 75th percentile in DEHP metabolite concentrations for a standard subject (34 years old, non-smoker with BMI of 28 kg/m2). Error bars indicate the 95% confidence intervals.