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Exposure Assessment of Prepubertal Children to Steroid Endocrine Disruptors. 2. Determination of Steroid Hormones in Milk, Egg, and Meat Samples

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In the present study, the occurrence of the main sex steroid hormones in milk, egg, and meat was evaluated on the basis of a highly specific gas chromatography−tandem mass spectrometry measurement method. Globally, the results indicated that targeted estrogens and androgens occurred at similar levels (concentration levels in the 10–100 ng kg^{-1} range) in the analyzed muscle and milk samples. The same compounds occurred at about 10-fold higher concentrations (i.e., in the 100–1000 ng kg^{-1} range) in eggs and kidney samples. More precisely, egg and milk appeared as a non-negligible sources of estradiol (i.e., 2.2 ± 0.8 and 3.1 ± 2.0 ng day^{-1}, respectively), whereas testosterone exposure is caused by ingestion of meat and/or egg (i.e., 12.2 ± 48.2 and 5.2 ± 2.3 ng day^{-1}, respectively). The provided exposure data will be further exploited in the scope of a risk assessment study regarding endocrine disruption associated with these molecules.

KEYWORDS: Endocrine disruptors; steroids; estrogens; androgens; milk; egg; tissues; dietary intake

INTRODUCTION

Global concern has been raised in recent years over the adverse effects resulting from exposure to chemical substances that can interfere with the endocrine system, so-called endocrine-disrupting chemicals (EDCs). The range of compound classes that have been pointed out in the field of endocrine disruption is extremely wide. Thus, examples of involved molecules belong to environmental contaminants (persistent organic pollutants such as dioxins, polychlorobiphenyls, or brominated flame retardants) (*1*), residues of phytosanitary products (organochlorine pesticides) (*2, 3*), phthalates (*4, 5*), or naturally occurring phytoestrogens (*6, 7*). However, steroid hormones are commonly considered as reference substances in terms of endocrine disruption, as they largely remain the most biologically active compounds. In particular, the activity of most estrogenic EDCs is usually expressed by comparison to the activity of estradiol, with values commonly found between 10^2- and 10^5-fold lower. These substances should, therefore, be included in the discussion related to endocrine disruption. In addition, it is assumed that estradiol acts as a growth factor in promoting cancer; an increase of estrogen-dependent diseases, such as testicular, breast, prostate, and ovarian cancers, has been recognized (*8*). Moreover, most recently, new findings suggest that some of its metabolites may also initiate mutations (*9, 10*). In this general context, a more specific issue in relation with endocrine disruption is related to low-dose effects and long-term exposure consequences, especially for specific populations at critical stages of development (fetus, newborn, prepubertal children). Indeed, several animal studies have shown that perinatal exposure to steroid hormones can produce adverse effects on male and female reproductive development (*11–14*). Moreover, cases of accidental exposures of children to estrogens have shown that children are sensitive to exogenous hormones (*15, 16*). Gynecomastia was observed in three prepubertal boys because of indirect exposure to estrogen cream used by their postmenopausal mothers.

Despite this toxicological relevance, a certain lack of precise and valid data regarding natural steroid hormones in food may be deplored, even though this route of exposure is recognized to be a major one for humans. Different authors have reviewed most papers dealing with the natural occurrence of sex steroid hormones in food products (*17–19*). They noted that pork, meat products, fish, and poultry contain similar amounts of steroids as cattle (in the nanograms per kilogram range). They observed that milk products and eggs seem to be an important source of steroids. Nevertheless, it must be noted that concentrations reported for various animal tissues were mainly determined by RIA and that problems in measuring low levels of steroids precisely have been recognized by studies showing high variation in the concentrations obtained between different assay methods and different laboratories (*20, 21*). From these scarce data, it can be argued that a more extended assessment of dietary exposure to natural sex steroids (especially from milk, egg, and meat) by confirmatory MS techniques appears today justified

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Table 1. Detailed Occurrence Levels Measured (Nanograms per Liter) for both Androgens and Estrogens in the Different Analyzed Milk Samples

		androgens						estrogens					
		free epitestosterone	free testosterone	DHEA	epitestosterone	testosterone	androstenedione	free estrone	free β-estradiol	free α-estradiol	estrone	β-estradiol	α-estradiol
whole milk	no. of quantified samples	8	8	8	7	7	8	8	8	8	8	8	7
	mean	87.8	4.1	119.8	99.3	9.0	876.3	14.1	3.0	1.2	187.1	13.5	31.7
	median	92.3	3.7	104.1	102.1	9.6	897.8	13.4	2.8	0.9	184.2	10.1	31.0
	min	46.5	1.6	38.3	51.3	4.6	550.5	5.1	1.3	0.0	115.7	8.0	25.1
	max	104.1	7.1	258.4	134.1	14.4	1159.7	22.4	7.0	3.3	260.4	25.1	43.1
	SD	18.9	2.3	71.7	26.4	3.8	215.9	5.6	1.8	1.1	45.4	7.5	6.4
	% conjugates				13	60					93	81	96
half skim milk	no. of quantified samples	17	17	17	17	17	17	16	16	16	17	17	17
	mean	50.0	3.2	57.4	70.8	9.9	421.9	7.2	2.6	1.4	141.1	13.0	30.1
	median	52.1	3.1	63.8	69.3	9.5	387.4	6.8	1.7	1.2	141.5	8.3	31.8
	min	22.2	0.7	14.0	31.3	3.4	213.6	2.5	0.8	0.0	77.5	5.5	19.6
	max	77.7	6.1	110.1	97.0	20.4	667.7	12.3	10.7	3.7	243.1	44.3	39.9
	SD	16.4	1.4	30.3	18.6	4.7	147.0	3.3	2.7	1.0	50.6	11.4	6.9
	% conjugates				30	66					94	79	95
skim milk	no. of quantified samples	12	11	12	12	11	12	12	12	12	12	12	12
	mean	36.4	3.2	40.9	51.0	7.8	289.8	6.0	1.5	1.2	207.2	16.6	40.3
	median	36.1	3.1	39.8	51.6	6.3	289.2	6.1	1.6	0.9	208.6	10.7	37.2
	min	24.0	1.5	15.4	34.4	2.9	186.5	1.2	0.5	0.1	58.5	5.7	23.3
	max	49.9	5.1	70.2	70.9	13.8	400.6	12.3	2.6	3.4	397.2	64.6	63.6
	SD	7.8	1.1	19.2	10.4	3.9	79.1	3.1	0.6	1.0	122.7	17.7	12.7
	% conjugates				30	52					96	87	97

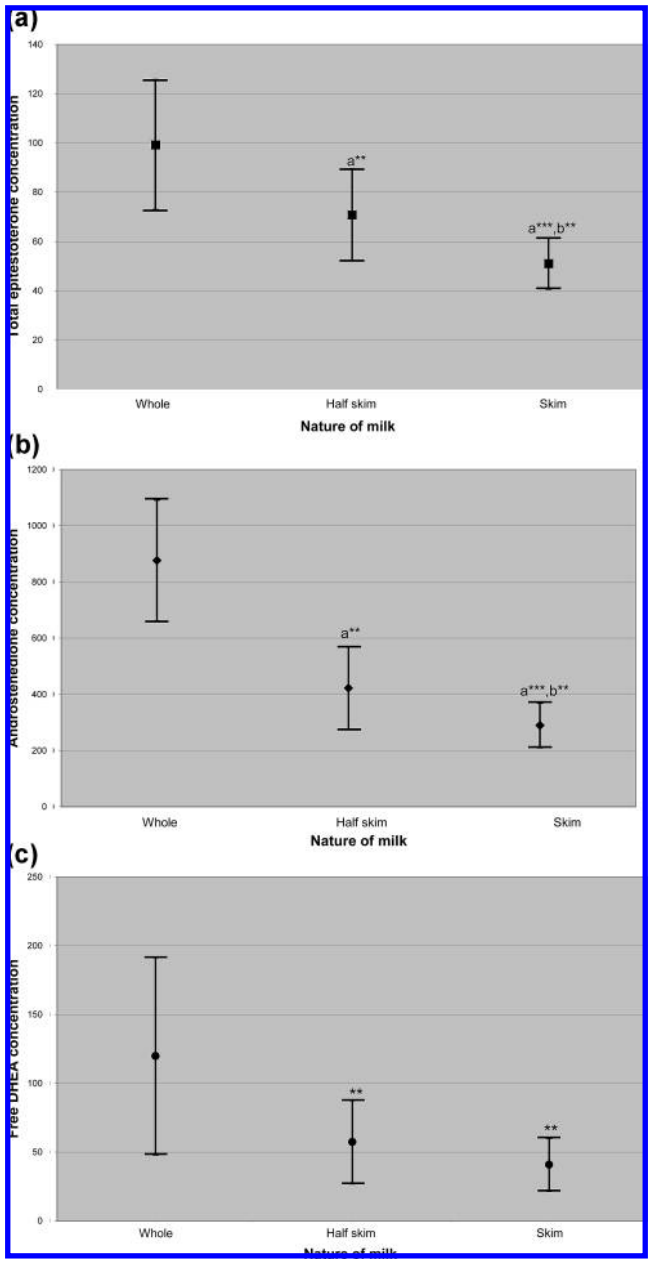

Figure 1. Concentrations of (**a**) epitestosterone, (**b**) and rostenedione, (**c**) free DHEA according to the nature of milk. Significant differences (Fisher's test) are labeled with asterisks (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

in the scope of (re)-evaluating the potential impact of these compounds on human health (*17, 22*).

In this context, the final purpose of the present study is to investigate to what extent steroid hormones in food may represent a risk of endocrine disruption for prepubertal children, considering the extremely low endogenous production of natural estrogens typically observed for this population. Thus, previously developed analytical methods based on gas chromatography coupled to tandem mass spectrometry were used for steroid measurement at ultratrace level in milk, egg (*23, 24*), and meat (*25*) samples. The present paper presents the quantification results obtained in these samples for the monitored estrogen- and androgen-related compounds (i.e., 5β-androstan-3-one-17β-ol, 5-androsten-3β,17α-diol, androsterone, 5α-androstan-3β,17α-diol, etiocholanolone, androstenedione, DHEA, 17α-testosterone, 17β-testosterone, 17α-estradiol, 17β-estradiol, and estrone). A first interpretation of these data in terms of risk assessment is also provided, in connection with existing JECFA and FDA recommendations regarding maximal acceptable daily intake for estradiol.

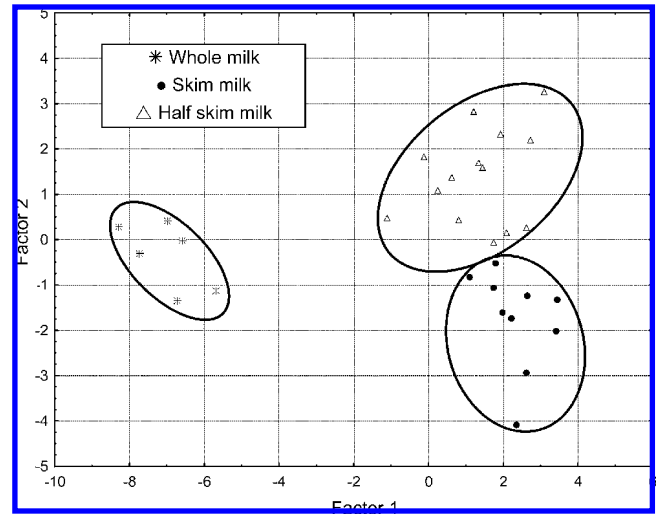

Figure 2. Results of the linear discriminant analysis showing the discrimination of the three groups of milk on the basis of their steroid metabolic profiles.

MATERIALS AND METHODS

Reagents and Chemicals. All solvents and reagents were of analytical or HPLC grade quality and purchased from Solvent Documentation Synthesis (SDS, Peypin, France). All SPE (ChromP, SiOH) were single-use cartridges also provided by SDS. Purified *Helix pomatia* enzymatic preparation was used for steroid deconjugation (Sigma, St. Louis, MO). Derivatization reagents *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and pentafluorobenzylbromide (PFBBr) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) and trimethyliodosilane (TMIS) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Standard reference steroids were purchased from Sigma (St. Louis, MO). Deuterated internal standards (etiocholanolone-d_5, 5α-androstane-3α,17β-diol-d_3, 4-androstenedione-d_3, 17α-testosterone-d_3, 17α-methyltestosterone-d_3, and 17β-estradiol-d_3) were provided by Steraloids (Wilton, NY).

Samples. Milk ($n = 37$) and egg samples ($n = 35$) were commercial products randomly collected from a French supermarket in 2006 and 2007. Meat samples ($n = 160$) were collected during two European projects of the fourth and fifth Framework Program (namely, EUROESTR and ISOSTER, respectively). Samples from nontreated animals (from different age, sex, and uncontrolled physiological state) were collected during the two previous projects. Samples from treated animals (from identical age, sex, . . .) were collected during the EUROESTR project. Steers were implanted with RevalorS and slaughtered 3 months after treatment.

Sample preparation. The sample preparation procedure (extraction and purification) used for efficient isolation of the target steroid fractions has been previously described (*23–25*).

Briefly, for milk and egg samples, an enzymatic hydrolysis with *H. pomatia* is performed at 52 °C during 15 h [conditions were optimized during a previous study (*26*)] before extraction of nonpolar compounds with diethyl ether. A first purification step is performed on a Chrom P SPE cartridge. Then, a liquid/liquid partitioning is performed with *n*-pentane to separate androgens and estrogens. Both fractions are finally purified on a silica SPE cartridge. A semipreparative HPLC is done as an ultimate purification step on both fractions for the milk sample and on the estrogen fraction for the egg sample.

For meat samples, a first liquid/liquid extraction with a methanol/acetate buffer mixture is performed. After an enzymatic hydrolysis for deconjugation of glucuronide metabolites, a first purification step is performed on a Chrom P SPE cartridge. A liquid/liquid partitioning is performed with *n*-pentane to separate androgens, estrogens, and sulfate

Table 2. Detailed Occurrence Levels Measured (Micrograms per Kilogram) for both Androgens and Estrogens in the Different Analyzed Egg Samples

	androgens							estrogens		
	5β-androstan-3-one-17β-ol	androsterone	etiocholanolone	free DHEA	androstenedione	epitestosterone	testosterone	17α-estradiol	17β-estradiol	estrone
no. of quantified samples	33	34	34	24	34	34	34	33	35	34
mean	3.59	0.21	36.03	0.34	28.10	1.46	0.86	0.24	0.36	0.93
median	3.35	0.21	34.22	0.30	27.83	1.35	0.77	0.18	0.34	0.93
min	1.26	0.08	21.56	0.12	9.42	0.73	0.16	0.03	0.15	0.15
max	7.60	0.40	65.14	0.70	48.33	2.72	1.88	0.72	0.99	2.47
SD	1.72	0.06	10.12	0.17	8.96	0.51	0.38	0.19	0.16	0.48
% conjugates	~0–5	~20–30	~0–5			~5–15	~5–15	~70–80	~75–85	~10–20

compounds. Androgens and estrogens are finally purified onto silica SPE cartridge, whereas sulfate compounds are first passed through a C_{18} SPE column; a solvolysis is then performed with an ethyl acetate/H_2SO_4 mixture, and finally the extract is purified on a silica SPE cartridge.

Derivatization Reaction. Derivatization of the androgen fraction was carried out with 20 μL of a MSTFA/TMIS/DTT mixture (1000:5:5, v/v/w) and incubation during 40 min at 60 °C. For milk and egg samples, the halogenated PFBBr/BSTFA reagent was preferred for derivatization of the estrogen fraction, because of particular benefit (in terms of specificity and sensitivity) when negative chemical ionization is used (*23*). For meat samples, derivatization was performed with MSTFA/TMIS/DTT for both estrogen and androgen fractions. Finally, both fractions were injected onto the GC-MS/MS system (splitless mode, 2 μL injected).

GC-MS/MS Measurement. Estrogen measurements (for milk and egg samples) were carried out by GC-MS/MS with negative chemical ionization (NCI) using methane as reagent gas. Electron energy was set at 100 eV. A HP-6890 gas chromatograph was coupled to a VG-QuattroII or QuattroMicro GC triple-quadrupole device (Waters-Micromass, Manchester, U.K.). Injector and transfer line temperatures were set at 250 and 280 °C, respectively. Source and analyzer temperatures were set to 280 and 100 °C, respectively. The GC column was a 30 m × 0.25 mm i.d., film thickness = 0.25 μm, ZB-5MS (Zebron). The temperature program was set as follows: 120 °C (2 min), 15 °C min^{-1} until 280 °C (0 min), 5 °C min^{-1} until 320 °C (6 min). Helium (N55) was used as carrier gas at 1 mL min^{-1}. Androgen measurements were carried out using the same GC-MS/MS equipment. However, positive electron ionization mode was preferred for this class of steroid. Electron energy was set at 70 eV. The temperature program was set as follows: 120 °C (2 min), 10 °C min^{-1} until 235 °C (3 min), 1 °C min^{-1} until 240 °C (0 min), and 5 °C min^{-1} until 300 °C (4 min). For the two analyzed fractions, the mass spectrometer was operated in the selected reaction monitoring (SRM) acquisition mode. Argon was used as collision gas at 2.8×10^{-3} mbar in the cell.

RESULTS

Milk Samples. For all analyzed milk samples ($n = 37$), the total hormone concentration levels (free plus deconjugated forms) were determined (enzymatic hydrolysis of the glucuronide and sulfate phase II metabolites). The detailed occurrence levels observed for androgens and estrogens monitored in these different samples are presented in **Table 1**. Considering that commercial milks are in fact mixtures of milk collected from different animals, the great variability observed in terms of concentrations for some compounds is not really surprising.

Estrone (E1) and 17α-estradiol (αE2) were measured at global mean concentration levels of 172 ± 84 and 34 ± 10 ng L^{-1}, respectively. These two compounds were found to be present mainly as conjugated forms (around 95%). 17β-Estradiol was found to be in lower concentration than E1 and αE2 (total estradiol content = 14 ± 13 ng. L^{-1}), with also a lower proportion of conjugated forms (nearly 80%). Due to this relatively high proportion of hydrophilic conjugated species, it can be argued that estrogens occur in milk essentially in the aqueous fraction. This hypothesis is confirmed by the fact that no significant relationship was found between the measured concentrations and the global fat content of the analyzed milk samples (i.e., skimmed, semiskimmed, or full cream).

In contrast, the concentration levels measured for the androgenic α-testosterone (αT), 4-androstenedione (AED), and free dehydroepiandrosterone (DHEA) appeared to be significantly correlated to the milk fat content (**Figure 1**). Thus, concentration levels of total epitestosterone (free + conjugated) are divided by a factor of 2 from whole milk (WM) to skim milk (SM) (mean values of 99 ± 26 and 51 ± 10 ng L^{-1}, respectively, $p < 0.0001$). The same observation was made for 4-androstenedione and free DHEA with mean concentration values in WM versus SM equal to 876 versus 290 ng L^{-1} ($p < 0.0001$) and 120 versus 41 ng L^{-1} ($p = 0.0018$), respectively. Nevertheless, one exception was pointed out for testosterone, for which the proportion of conjugated phase II metabolites was estimated to about 60% (whereas this proportion for epitestosterone was estimated to be only 26%). As a consequence, no major relationship was found between the observed concentrations of testosterone and milk fat content.

A linear discriminant analysis (LDA) was then performed on the global exposure data set. Statistical variables corresponded to the concentrations determined for free and total steroid hormones, as well as the relative proportions of free and conjugated forms. Two canonical variables (discriminant axis) were extracted by this approach, as shown in **Figure 2**. With respect to the above observations, the three types of analyzed milk samples (i.e., skimmed, semiskimmed, and full cream) were confirmed to be statistically different in terms of steroid profile, although the half-skim milk group remained relatively close to the skim milk group.

Egg Samples. For all analyzed egg samples ($n = 35$), the sum of conjugated and free hormones was determined. The detailed occurrence levels for androgens and estrogens monitored in these samples (egg yolks) are presented in **Table 2**.

E1 appeared as the major estrogen quantified in these samples (mean value of 0.93 ± 0.48 μg kg^{-1}) and was found mainly as the unconjugated form. α-E2 and β-E2 were also quantified in the same samples, at 0.24 ± 0.19 and 0.36 ± 0.16 μg kg^{-1}, respectively. In opposition with the previous results obtained for E1, αE2 and β E2 were found mainly to be conjugated (70–85%). The presence of a ketone function would influence the phase II metabolite distribution in egg samples.

With regard to androgens, etiocholanolone and 4-androstenedione appeared as the two major androgens quantified in egg samples (mean concentration levels of 36.0 ± 10.1 and 28.1 ± 9.0 μg kg^{-1}, respectively). 17α- and 17β-testosterones were estimated at lower concentration levels (mean values of 1.46 ± 0.51 and 0.86 ± 0.38 μg kg^{-1}, respectively). In contrast with

Table 3. Detailed Occurrence Levels Measured (Micrograms per Kilogram) for both Androgens and Estrogens in the Different Analyzed Meat Samples

			androgens								estrogens	
			5-androsten-3β-17α-diol	5α-androstane-3β,17α-diol	androsterone	etiocholanolone	DHEA	epitestosterone	androstendione	testosterone	17α-estradiol	17β-estradiol
muscle	control group	no. of quantified samples	70	70	70	70	70	70	70	70	70	70
		mean	0.23	0.02	0.07	0.05	0.30	0.06	0.14	0.23	0.00	0.01
		median	0.19	0.01	0.05	0.04	0.25	0.03	0.10	0.01	0.00	0.00
		min	0.04	0.00	0.01	0.01	0.09	0.01	0.00	0.00	0.00	0.00
		max	0.75	0.48	0.21	0.40	1.29	0.43	1.61	7.15	0.00	0.07
		SD	0.15	0.06	0.04	0.06	0.21	0.06	0.21	0.91	0.00	0.01
	treated group (E + Tb)	no. of quantified samples	16	16	16	16	16	16	16	16	16	16
		mean	0.22	0.03	0.04	0.05	0.37	0.05	0.04	0.02	0.06	0.09
		median	0.22	0.02	0.04	0.02	0.21	0.04	0.04	0.02	0.01	0.04
		min	0.12	0.00	0.02	0.01	0.11	0.02	0.00	0.00	0.00	0.02
		max	0.33	0.16	0.06	0.25	2.76	0.09	0.08	0.05	0.83	0.61
		SD	0.06	0.04	0.01	0.08	0.64	0.02	0.02	0.01	0.20	0.15
kidney	control group	no. of quantified samples	45	45	45	45	45	45	45	45	45	45
		mean	3.44	1.18	0.25	2.13	0.88	0.70	0.50	1.49	0.21	0.04
		median	2.85	1.00	0.19	1.25	0.73	0.36	0.33	0.54	0.07	0.01
		min	1.44	0.26	0.03	0.18	0.40	0.01	0.01	0.01	0.00	0.01
		max	15.96	3.52	0.99	11.04	2.05	6.91	2.96	13.91	3.97	0.54
		SD	2.38	0.74	0.22	2.39	0.40	1.11	0.54	2.81	0.60	0.09
	treated group (E + Tb)	no. of quantified samples	13	13	13	13	13	13	13	13	13	13
		mean	3.16	0.88	0.07	0.43	1.02	0.38	0.13	0.12	0.68	0.20
		median	3.29	0.85	0.06	0.44	1.01	0.36	0.14	0.13	0.62	0.18
		min	1.44	0.50	0.04	0.17	0.50	0.06	0.05	0.01	0.03	0.01
		max	5.35	1.43	0.10	0.63	1.71	0.93	0.20	0.20	1.70	0.57
		SD	0.98	0.32	0.02	0.13	0.41	0.20	0.05	0.06	0.47	0.15
liver	control group	no. of quantified samples	5	5	5	5	5	5	5	5	5	5
		mean	5.86	1.10	0.15	0.42	1.04	0.09	0.00	0.21	0.00	0.00
		median	6.45	0.70	0.13	0.39	1.11	0.05	0.00	0.20	0.00	0.00
		min	3.81	0.26	0.05	0.10	0.59	0.01	0.00	0.16	0.00	0.00
		max	7.23	2.50	0.28	0.90	1.46	0.24	0.01	0.27	0.01	0.00
		SD	1.40	0.89	0.09	0.29	0.39	0.09	0.00	0.05	0.00	0.00
	treated group (E + Tb)	no. of quantified samples	11	11	11	11	11	11	11	11	11	11
		mean	4.74	0.84	0.24	0.56	1.13	0.10	0.00	0.22	0.39	0.06
		median	4.57	0.74	0.16	0.49	0.93	0.07	0.00	0.23	0.39	0.04
		min	2.73	0.31	0.01	0.00	0.49	0.02	0.00	0.08	0.06	0.00
		max	7.26	1.47	0.73	1.44	2.62	0.23	0.02	0.35	0.87	0.17
		SD	1.50	0.40	0.20	0.39	0.62	0.08	0.01	0.08	0.26	0.07

Table 4. Testosterone (**A**) and Estradiol (**B**) Dietary Intake Corresponding to 1.5 L of Milk + 100 g of Egg + 300 g of Muscle + 100 g of Liver + 50 g of Kidney + 50 g of Tissue Fat

(**A**) 17β-Testosterone

	range (ng day^{-1})	mean (ng day^{-1})	% ADI (120000 ng day^{-1})[a]	% max secure daily intake (320 ng day^{-1})[b]
milk	4.3–30.5	13.7	0.01	4.3
egg[c]	8.0–94.0	43.0	0.04	13.4
muscle	0.0–2145.0	69.0	0.06	21.6
kidney	0.5–695.5	74.5	0.06	23.3
liver	16.0–27.0	21.0	0.02	6.6
tissue fat			no data available	
total food intake		221.2	0.18	69.1

(**B**) 17β-Estradiol

		range (ng day^{-1})	mean (ng day^{-1})	% ADI (3000 ng day^{-1})[a]	% max secure daily intake (65 ng day^{-1})[b]
	milk	8.3–96.9	21.5	0.7	33.1
	egg[c]	7.5–44.5	18.0	0.6	27.7
muscle	from control	0.0–21.0	3.0	0.1	4.6
	from treated (1 implant)	4.8–6.9	6.0	0.2	9.2
	from treated (2 or 4 implants)	9.0–186.0	33.0	1.1	50.8
kidney	from control	0.5–27	2.0	0.07	3.1
	from treated (1 implant)	0.5–8.5	3.5	0.12	5.4
	from treated (2 or 4 implants)	9.0–28.5	14.0	0.47	21.5
liver	from control	0.0	0.0	0.0	0.0
	from treated (1 implant)	0.0–4.0	2.0	0.06	3.0
	from treated (2 or 4 implants)	0.0–17	8.0	0.27	12.3
tissue fat[d]	from control	0.0	0.0	0.0	0.0
	from treated (1 implant)	0.0–3.5	1.5	0.05	2.3
	from treated (2 or 4 implants)	4.5–8.5	7.0	0.23	10.7
total food intake	if meat is from nontreated		44.5	1.5	68.5
	if meat is from treated (1 implant)		52.5	1.75	80.8
	if meat is from treated (2 or 4 implants)		101.5	3.4	156.2

[a] Calculated on the basis of JECFA's ADI for a person weighing 60 kg. [b] Maximum secure daily intake of FDA. [c] We considered 50 g of eggs in this calculation as steroid concentrations were determined only in egg yolk. [d] Data from ref *33*.

Table 5. Consumption Data (Grams per Person per Day) for Children from INCA Study (*28*)

product	mean (g)
milk	219
muscle	53
liver/kidney	2
egg	12 (6)

milk samples, where free DHEA appeared as a predominant androgen, in egg samples, it occurs as a minor one (mean value at 0.34 ± 0.17 μg kg^{-1}). As observed for estrone, free and total androgen levels appeared in the same order of magnitude. Consequently, androgens occur in egg mainly as unconjugated form.

Egg samples were issued from two different production processes, that is, open area versus intensive battery production. Whereas concentration levels found for androgens are independent of this factor, significant differences according to this parameter were noticed for estrogens. Thus, concentration levels of βE2 and E1 in open area versus intensive battery samples were 0.32 versus 0.44 μg kg^{-1} ($p = 0.034$) and 0.76 versus 1.13 μg kg^{-1} ($p = 0.049$), respectively.

Meat Samples. The occurrence of steroid hormones in meat samples (muscle, kidney, and liver) collected from control and anabolized animals [treated with RevalorS implants (one, two, or four) containing 24 mg of estradiol and 120 mg of trenbolone] was previously evaluated in the laboratory within the framework of two European projects of the fourth and fifth Framework Programs (namely, EUROESTR and ISOSTER, respectively). The main results obtained in these studies are summarized in **Table 3**.

On the basis of these results, muscle, liver, and particularly kidney may appear to be non-negligible sources of both androgens and estrogens. The main androgen compounds quantified in tissues samples from control animals were 5-androstene-3β,17α-diol and etiocholanolone in kidney (mean values of 3.44 ± 2.38 and 2.13 ± 2.39 μg kg^{-1}, respectively), DHEA and 17β-testosterone in muscle (0.30 ± 0.21 and 0.23 ± 0.91 μg kg^{-1}, respectively), and 5-androstene-3β,17α-diol and 5α-androstane-3β,17α-diol in liver (5.86 ± 1.40 and 1.10 ± 0.89 μg kg^{-1}, respectively). The standard deviation (SD) of some compounds in matrices from control animals can appear to be relatively high. Indeed, no particular effort was made to reduce interindividual variability (animals were from different ages, sexes, and uncontrolled physiological states). In treated animals, androgen concentrations decreased in these matrices except in liver, where they remained at similar levels.

With regard to estrogens, 17β-estradiol was found in very weak concentrations in control animals, that is, near the detection limit of the method used (LOD = 0.01 μg kg^{-1}). It can be noted that after anabolic treatment, residues of βE2 increased by a

Table 6. Testosterone (**A**) and Estradiol (**B**) Dietary Intake According to Chidren Consumption of Milk, Meat, and Eggs

(**A**) 17β-Testosterone

	mean (ng day^{-1})	% ADI (40000 ng day^{-1})[a]	% max secure daily intake (320 ng day^{-1})[b]
milk	2.0	0.005	0.6
egg[c]	5.2	0.01	1.6
muscle	12.2	0.03	3.8
kidney/liver	3.0/0.4	0.007	0.9/0.1
total food intake	22.4/19.8	0.06	7.0/6.2

(**B**) 17β-Estradiol

		mean (ng day^{-1})	% ADI (1000 ng day^{-1})[a]	% max secure daily intake (65 ng day^{-1})[b]
	milk	3.1	0.31	4.8
	egg[c]	2.2	0.22	3.4
muscle	from control	0.5	0.05	0.7
	from treated (1 implant)	1.0	0.1	1.5
	from treated (2 or 4 implants)	5.8	0.58	8.9
kidney/liver	from control	0.08/0	<0.01	0.1
	from treated (1 implant)	0.14/0.04	0.01	0.3
	from treated (2 or 4 implants)	0.52/0.18	0.05/0.02	0.9
total food intake	if meat is from nontreated	5.9	0.59	9.1
	if meat is from treated (1 implant)	6.44/6.34	0.64	9.9
	if meat is from treated (2 or 4 implants)	11.62/11.28	1.16	17.9

[a] Calculated on the basis of JECFA's ADI for a child weighing 20 kg. [b] Maximum secure daily intake of FDA. [c] We considered 6 g of eggs in this calculation as steroid concentrations were determined only in egg yolk.

factor of 5 in kidney (0.04 vs 0.20 μg kg^{-1}, $p < 0.0001$) and by a factor of about 10 in muscle (0.01 vs 0.09 μg kg^{-1}, $p < 0.0001$). It was not possible to quantify βE2 in liver in animals from the control group. Indeed, estrogen concentrations increased after treatment, αE2 being estimated at 0.39 ± 0.26 μg kg^{-1} and 17β-estradiol at 0.06 ± 0.07 μg kg^{-1}. Once more, the SD can appear to be quite elevated. Nevertheless, animals were treated either with one, two or four implants and therefore it can be assumed that residue levels from a one-implant animal will be different from those from a four-implant one.

DISCUSSION

Exogenous Exposure to Natural Sex Steroid. On the basis of the previous findings related to the concentration levels of steroid hormone in various food products, a first interpretation was made in the scope of evaluating the potential risk associated with these endocrine-disrupting chemicals for human health, especially for critical populations such as prepubertal children. Then, a theoretical food intake, based on the concentration levels determined for 17β-estradiol and 17β-testosterone, was compared to (1) the acceptable daily intake (ADI) established by the JECFA (*27*) for 17β-testosterone and 17β-estradiol (equivalent to 2 μg kg^{-1} of body weight per day and 50 ng kg^{-1} of body weight per day, respectively) and (2) the maximum secure daily intake established by the FDA (*28*) for 17β-testosterone and 17β-estradiol (equivalent to 320 and 65 ng day^{-1}, respectively), these limits corresponding to 1% of the endogenous levels produced by the segment of the population with the lowest daily production, that is, prepubertal girls and boys, respectively.

We are conscious of the extremely high difference in terms of ADI's values existing between these two systems of reference. Nevertheless, those two systems are well acknowledged, and we took care to compare the theoretical food intake to both of them.

It is assumed that a person with a body weight of 60 kg consumes every day over an adult lifetime 500 g of meat whatever the considered species (this theoretical intake consists of 300 g of muscle, 100 g of liver, 50 g of kidney, and 50 g of fat), as well as 1.5 L of milk, 100 g of egg, and 20 g of honey (*27*). The corresponding food intakes for 17β-testosterone and 17β-estradiol, estimated from our exposure results obtained in milk, egg, and meat samples, are presented in **Table 4**.

With regard to 17β-testosterone, eggs, muscle, and kidney represent the main sources of this steroid (exogenous intake estimated to 43.0, 69.0, and 74.5 ng day^{-1}, respectively). For this compound, it can then be concluded that none of the maximum daily intake values recommended by either the JECFA or FDA is reached. However, it must be kept in mind that in this calculation mode, only testosterone is pointed out, even though other androgens (testosterone precursors and metabolites) are contributing to the global real activity.

As far as estrogens are concerned, it can be observed that the main sources of estradiol are milk and eggs (exogenous intake estimated to 21.5 and 18.0 ng day^{-1}, respectively) and, to a minor extent, meat from untreated animals. Nevertheless, when meat from treated animals is considered, it leads to an increase of almost a factor 3 of the daily intake from this matrix. Indeed, consumption of meat from nontreated animals leads to a food intake of estradiol of around 5 ng day^{-1}, whereas it reaches almost 13 ng day^{-1} with meat from treated animals (one implant). Moreover, when Good Veterinary Practice (GVP) is not followed (two or four implants), the resulting intake (i.e., 62.0 ng day^{-1}) reaches the same order as the one induced by milk and egg consumption (i.e., 39.5 ng day^{-1}).

With regard to these results, JECFA's ADI for estradiol is never reached whatever the meat origin (1.5−3.4% of the ADI), whereas estradiol supply almost reaches 70% of the FDA maximum secure daily intake. Furthermore, in the case of consumption of meat from treated animals (when GVP is not followed), the resulting total food intake crossed this limit and is twice that calculated when meat from nontreated animals is considered.

Case of Prepubertal Children. The food consumption levels established by the JECFA in leading a risk assessment can appear to be very conservative in the case of prepubertal children. Our objective is to evaluate the potential risk associated with steroid hormones for this critical population. Therefore, with respect to children consumption data (**Table 5**) determined in the course of a national investigation performed by AFSSA, CREDOC, and DGAL (*29*), estradiol and testosterone theoretical food intakes have been (re)calculated (**Table 6**).

Testosterone is mainly provided by meat ingestion (i.e., 12.2 ng day^{-1}), whereas milk appears to be the most significant source of estradiol (i.e., 3.1 ng day^{-1}). Maximum secure daily intake of FDA does not seem to be reached by consumption of products such as milk, meat, and eggs. Indeed, estradiol supply represents 9.1−17.9% of the ADI according to the meat origin. However, in this calculation, other food products such as cheese, butter, or fish have not been taken into account, but even so contribute to the global dietary intake of steroid hormones for children. Indeed, the 17β-estradiol concentration in fresh cheese has been evaluated to be 11 ng kg^{-1}, that in ripened cheese to be 25 ng kg^{-1}, and that in butter to be 82 ng kg^{-1} (*30, 31*). In fish, concentrations are supposed to be of the same order of magnitude as those of mammalian tissues (*18*). Assuming that children consume every day 21 and 23 g of fish and cheese, respectively (*29*), it would lead to an increase of 0.4 ng day^{-1} in the corresponding food intake. Moreover, a recent review dealing with the determination of the maximum daily production of estradiol in prepubertal boys indicates that this production could be significantly lower than commonly admitted (*17*). Indeed, such measurements of extremely low concentrations of estradiol were traditionally performed with RIA, but the available results present huge variability, and a clear uncertainty remains about the reality of these circulating levels of natural estrogens in the blood of prepubertal children. As a consequence, several studies have highlighted the necessity to (re)evaluate these concentrations using new measurements based on a confirmatory MS technique. We have already initiated this work (Courant et al., submitted paper) and confirmed that endogenous levels of estrogens in prepubertal children are lower than reported in the literature (*32*). Consequently, for this critical population, even a small exogenous intake would account for a major change in the total activity of the involved hormone (*22*).

The next issue of this study will be to investigate the chemical forms (balance between free and glucurono- and sulfo-conjugates) of estrogens occurring in food and to take into account their bioactivity and bioavailability. In addition, 17β-estradiol fatty acid esters have been detected in bovine tissues (*33, 34*). As these compounds are suspected to participate in the final estrogenic activity resulting from food intake, the determination of these lipoidal esters in milk and especially in eggs will merit investigation as well.

ABBREVIATIONS USED

ADI, acceptable daily intake; AED, 4-androstenedione; DHEA, dehydroepiandrosterone; E1, estrone; EDC, endocrine-disrupting chemicals; FDA, U.S. Food and Drug Administration; GC-MS/MS, gas chromatography−tandem mass spectrometry; GVP, Good Veterinary Practice; HPLC, high-performance liquid chromatography; JECFA, Joint Expert Committee on Food Additives; NCI, negative chemical ionization; RIA, radioimmunoassay; αE2, 17α-estradiol; βE2, 17β-estradiol.

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