Reproductive toxicology of endocrine disruptors

Effects of cadmium, phthalates and phytoestrogens on testicular steroidogenesis

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2008

ISBN: 978-91-7264-631-5
Printed by Arkitektkopia, Umeå, 2008

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ABSTRACT

A number of investigations during the last two decades describe adverse trends in male reproductive health, which have been proposed to be caused by environmental factors with endocrine disrupting properties. In contrast to many other toxicants, endocrine disruptors often do not show linear dose-response relationships typical of those found in traditional toxicological studies. For many compounds, low-dose exposure causes effects opposite to the ones seen after high-dose exposure. In addition, the timing of exposure has been found to be critical. Hence, to correctly assess the impact of endocrine disruptors on reproductive health requires in-depth knowledge of their mechanisms of action.

This thesis aimed at identifying the mechanisms underlying the effects of cadmium (Cd), phthalates and phytoestrogens on testicular steroidogenesis. For this purpose, *in vitro* as well as *in vivo* models were used. Cd was found to inhibit testosterone synthesis *in vivo* by down-regulating LH receptor gene expression and reducing the testicular levels of cAMP and StAR protein. In addition, Cd caused a pronounced increase in testicular prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), suggesting that Cd exerts its suppressive effect on steroidogenesis also by inducing the inhibitory PKC pathway. Pre-treatment with zinc (Zn) protected completely against Cd-induced effects on testosterone and $PGF_{2\alpha}$. Furthermore, we observed that Cd exposure increased glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in the testis. GAPDH is a potent coactivator of androgen receptor-mediated transcription and the up-regulation found in our study is probably a compensatory response to reduced testosterone concentrations. This finding is interesting since GAPDH has been proposed to have an important role in the regulation of apoptosis as well as sperm motility. We discovered that mono-(2-ethylhexyl) phthalate (MEHP), the active metabolite of the frequently used phthalate di-(2-ethylhexyl) phthalate (DEHP), stimulates Leydig cell steroidogenesis *in vitro*, by a cAMP- and StAR-independent mechanism. MEHP exposure caused a similar effect in granulosa cells. Gene expression analysis revealed that MEHP is likely to stimulate steroidogenesis by increasing the amount of cholesterol available for steroid synthesis. In the last investigation, we examined the effects of low-dose phytoestrogen exposure on testosterone synthesis during puberty in male goats. Isoflavones present in clover increased plasma concentrations of testosterone and free as well as total triiodothyronine (T_3). T_3 has previously been shown to induce testosterone synthesis and it is possible that an elevated T_3 secretion underlies the increased plasma testosterone levels.

Reduced fertility and reproductive tract malformations affect both the individual and the society. Hence, a sound knowledge of reproductive toxicants is of crucial importance. The findings presented in this thesis provide new insights into the reproductive toxicology of endocrine disruptors and may be valuable for risk assessment purposes.

Key words: Endocrine disruptors, reproductive toxicology, cadmium, phthalates, DEHP, MEHP, phytoestrogens, steroidogenesis, testosterone, Leydig cell

ABBREVIATIONS

All abbreviations are explained when they first appear in the text.

Note that AR is used as abbreviation for both adrenergic receptor (paper III) and androgen receptor.

LIST OF PAPERS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals (I-V).

I. **Gunnarsson D**, Nordberg G, Lundgren P, Selstam G. Cadmium-induced decrement of the LH receptor expression and cAMP levels in the testis of rats. Toxicology. 2003 Feb 1;183(1-3):57-63.

II. **Gunnarsson D**, Svensson M, Selstam G, Nordberg G. Pronounced induction of testicular $PGF_{2\alpha}$ and suppression of testosterone by cadmium-prevention by zinc. Toxicology. 2004 Jul 15;200(1):49-58.

III. **Gunnarsson D**, Nordberg G, Selstam G. Differential effects of cadmium on the gene expression of seven-transmembrane-spanning receptors and GAPDH in the rat testis. Toxicol Lett. 2007 Jan 10;168(1):51-7

IV. **Gunnarsson D**, Leffler P, Ekwurtzel E, Martinsson G, Liu K, Selstam G. Mono-(2-ethylhexyl) phthalate stimulates basal steroidogenesis by a cAMP-independent mechanism in mouse gonadal cells of both sexes. Reproduction. 2008 May;135(5):693-703.

V. **Gunnarsson D**, Selstam G, Ridderstråle Y, Holm L, Ekstedt E, Madej A. Effects of dietary phytoestrogens on plasma testosterone and triiodothyronine (T_3) levels in male goat kids. Manuscript.

Articles I-IV are reprinted with permission from the publishers.

Additional publications not included in the thesis

Liu L, Rajareddy S, Reddy P, Du C, Jagarlamudi K, Shen Y, **Gunnarsson D**, Selstam G, Boman K, Liu K. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development. 2007 Jan;134(1):199-209.

Toom A, Arend A, **Gunnarsson D**, Ulfsparre R, Suutre S, Haviko T, Selstam G. Bone Formation Zones in Heterotopic Ossifications: Histologic Findings and Increased Expression of Bone Morphogenetic Protein 2 and Transforming Growth Factors β2 and β3. Calcif Tissue Int. 2007 Apr;80(4):259-67.

INTRODUCTION

History and mechanisms of endocrine disruption

A functional endocrine system is essential for development, growth and reproductive functions in humans as well as wildlife. Hence, identifying substances that interfere with hormone synthesis and/or hormonal signalling is of crucial importance.

The first reports of substances adversely affecting endocrine functions came in the mid 20th century. Among the first compounds to be identified were the insecticide dichlorodiphenyltrichloroethane (DDT) and the drug diethylstilbestrol (DES). Burlington and Lindeman showed in 1950 that DDT interfered with the development of secondary sex characteristics in cockerels, whereas a number of studies in the 1960´s described how DDT exposure resulted in eggshell thinning and subsequent nesting failures in birds (Burlington & Lindeman 1950, Ratcliffe 1967, Bitman *et al.* 1969, Porter & Wiemeyer 1969). Whether the changes in eggshell thickness is caused by the estrogenic actions of o,p´-DDT or the inhibitory effects of the persistent metabolite p,p´-dichlorodiphenyldichloroethylene (p,p´-DDE) on prostaglandin synthesis is still under evaluation (Lundholm 1997, Holm *et al.* 2006). Today it is well established that DDT (i.e the different isomers and metabolites) have both estrogenic and antiandrogenic properties and that exposure may result in numerous reproductive tract malformations in animals of both sexes (Bornman *et al.* 2007). For this reason, DDT has been banned in most industrialized countries since long. However, it is still used in some developing countries against insect-borne diseases such as malaria.
In contrast to DDT, DES was specifically designed for its potent estrogenic activity and it was used by pregnant women to help prevent miscarriage. At the time when DES was introduced, in the late 1940´s, studies had shown that fetal death *in utero* was preceded by a premature decrease in estrogen level and DES was prescribed to restore the hormonal balance (Smith & Smith 1949a, 1949b). However, in 1971 Herbst and colleagues found that *in utero* exposure to DES was strongly associated with vaginal adenocarcinoma (Herbst *et al.* 1971). This study was followed by reports on DES-induced feminization (hypospadias, microphallus, cryptorchidism) in the male offspring and a reduction in female fertility (Giusti *et al.* 1995, Newbold 2004). Together, the investigations on reproductive tract development and fertility led not to only to the ban of DES but were also of general scientific value. DES was the first *in utero* estrogenic toxicant to be identified in humans and as such it revealed the

potentially toxic effects of estrogens and estrogen-like compounds (Newbold & Jeffersson 2005). Importantly, the discovery of detrimental reproductive effects caused by DDT and DES exposure in wildlife and humans demonstrated that also compounds with low acute toxicity could induce adverse health effects, by disrupting endocrine regulation. In addition, due to the widespread publicity these findings received, public concern was raised about the influence of toxicants on human and animal health.
Since the first discoveries of synthetic substances with estrogenic properties numerous other compounds, synthetic as well as natural, have been shown to affect the endocrine system by different mechanisms. Collectively, such substances are termed endocrine disrupting chemicals (EDCs), which is defined by The International Programme on Chemical Safety (IPCS) as "an exogenous substance or mixture that alters the functions(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (IPCS 2002). At present, apart from pesticides and pharmaceuticals, several heavy metals, industrial chemicals (e.g polychlorinated biphenyls, phthalates) and naturally occurring phytoestrogens are classified as EDCs.

EDCs may disturb endocrine functions by interfering with the synthesis, secretion and signalling of peptide as well as steroid hormones. The chlorotriazine herbicide atrazine is a good example of an endocrine disruptor which effects can be ascribed to inhibition of peptide hormone synthesis. In both male and female rats it reduces luteinizing hormone (LH) synthesis, by affecting hypothalamic control of this hormone, thereby causing a delayed pubertal onset (Cooper *et al.* 2000, Laws *et al.* 2000, Trentacoste *et al.* 2001). Biosynthesis of steroids, on the other hand, is often affected by direct effects on crucial steps in the synthesis pathway. For example, the fungicide ketoconazole; a potent inhibitor of adrenal as well as gonadal steroidogenesis, acts by selectively inihibiting steroidogenic p450 enzymes (Loose *et al.* 1983, Gal *et al.* 1994). Apart from reducing or stimulating steroid hormone production such compounds may change the androgen/estrogen ratio, thus influencing e.g pubertal development and prostate homeostasis (Marty *et al.* 1999, Bianco *et al.* 2006). Some endocrine disruptors instead disturb the release of hormones. Heavy metal ions, such as cadmium (Cd) and zinc (Zn), have the capacity to change basal as well as stimulated secretion of LH and prolactin from the pituitary (Cooper *et al.* 1987, Winstel & Callahan 1992).
Other substances, including previously mentioned DDT and DES, interfere with hormonal signalling. This is probably the most common mechanism of action of EDCs and today numerous chemicals have been identified as either agonists or antagonists of steroid hormone receptors, primarily the androgen

receptor (AR) and estrogen receptors (ERs). Initial concern was primarily over substances with estrogenic or anti-estrogenic activity, but during the last decades the awareness of compounds acting at the AR has grown. The pesticides linuron, vinclozolin and procymidone are all competitive AR antagonists that inhibit androgen-regulated gene expression (Gray *et al.* 2001). However, in the absence of the natural ligand dihydrotestosterone (DHT), vinclozolin as well as its two primary metabolites M1 and M2 act as partial AR agonists (M2>>vinclozolin>M1) (Wong *et al.* 1995, Molina-Molina *et al.* 2006), demonstrating that opposite effects could be induced by the same compound, depending on the amount of endogenous hormone. The fact that some chemicals show affinity for more than one hormone receptor also contributes to the complexity of EDCs. The metoxhychlor metabolite 2,2-bis(p-hydroxyphenol)-1,1,1-trichloroethane (HPTE) is both an ER agonist and an ERβ and AR antagonist, whereas vinclozolin antagonizes not only AR but also progesterone receptor (PR) and mineralcorticoid receptor (MR) action (Waters *et al.* 2001, Molina-Molina *et al.* 2006).

Endocrine disrupting effects can also be mediated through the aryl hydrocarbon receptor (AhR). The AhR is a ligand-activated transcription factor known to bind certain polcyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and induce the transcription of genes involved in the metabolism of xenobiotics (Bemanian *et al.* 2004). However, activation of AhR may also impair both estrogen-induced responses and steroidogenesis (Safe 1995, Aluru & Vijayan 2006). The effect on estrogen signalling is considered to be caused by AhR associating with ERα thereby decreasing ER-induced gene transcription, whereas steroid synthesis is inhibited by reduced expression of LH receptor, steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (p450scc) (Fukuzawa *et al.* 2004, Aluru & Vijayan 2006, Khan *et al.* 2006).

A number of less well-described modes of actions of EDCs may show to be of importance. Prostaglandins are important regulators of reproductive functions, i.e ovulation, luteolysis and implantation, but their role in endocrine disruption is only little studied (Olofsson *et al.* 1990, Goff 2004, Guillette 2006). Altered prostaglandin synthesis has been associated with DDT-induced eggshell thinning (see above) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is known to inhibit gonadal steroidogenesis by down-regulating StAR mRNA and protein expression (Chung *et al.* 1998, Fiedler *et al.* 1999). Other target sites for endocrine disruptors are proteasome-mediated degradation of steroid receptors and germ line DNA methylation (Tabb & Blumberg 2006). Epigenetic changes, such as DNA methylation, can be inherited and as a consequence fertility problems will be passed down to every subsequent generation. Anway and collaborators

showed in rats that *in utero* exposure (E8-E15) to vinclozolin or methoxychlor resulted in germ cell defects (sperm number and viability) that were transmitted to at least the F4 generation (Anway *et al.* 2005). The reduced spermatogenic capacity was detected in almost all males of the different generations and correlated with altered DNA methylation in the male germ line. In addition, it is likely that some effects of EDC exposure are caused by interactions with the newly discovered membrane steroid hormone receptors. Seven-transmembrane-spanning progestin and estrogen receptors have been identified in several vertebrate species (including humans), whereas studies in fish suggest the existence of a membrane androgen receptor (Thomas *et al.* 2006). Membrane steroid hormone receptors are G-protein coupled receptors that, in contrast to nuclear hormone receptors, mediate rapid non-genomic cellular responses (Carmeci *et al.* 1997, Thomas 2008). Many xenobiotics are known to have non-genomic effects and in 2006 Thomas and Dong demonstrated that a number of environmental estrogens, such as genistein and bisphenol A, could bind to the membrane estrogen receptor G-protein coupled receptor 30 (GPR30) and activate non-classical estrogen signalling pathways (Loomis & Thomas 2000, Walsh *et al.* 2005, Thomas & Dong 2006).

Trends in male reproductive health and the possible impact of endocrine disruptors

A number of investigations during the last two decades describe adverse trends in male reproductive health, which have been proposed to be caused by environmental factors. Observed changes include declining sperm concentrations associated with poor semen quality and an increased incidence of testicular cancer as well as reproductive tract malformations (i.e cryptorchidism and hypospadias). By using meta-analysis, Carlsen and colleagues could show that mean sperm concentrations among healthy men had decreased by over 50 percent (from 113 x 10^6/ml to 66 x 10^6/ml) during the 50-year period from 1940 to 1990 (Carlsen *et al.* 1992). Auger and co-workers noted in their study, of a French population, that the decline in sperm concentrations was accompanied by a reduction of sperm motility (Auger *et al.* 1995). In addition, a recent Danish study showed that ~40% of the men had sperm concentrations lower than 40 x 10^6/ml, which has been suggested as the lower limit for optimal fertility (Bonde *et al.* 1998, Andersson *et al.* 2008).
However, it is likely that geographical differences exist. For example, an analysis of semen parameters in men from the Seattle area revealed no downward trend in sperm concentrations from 1972 to 1993 (Paulsen *et al.*

1996). There is a considerable geographic variation also in the incidence of testicular cancer, but in a majority of developed countries it is likely to increase. Richiardi and his collaborators analyzed more than 27 000 testicular cancer cases from Northern European countries and found, apart from large regional differences, that the incidence is increasing (by 2.6-4.9% annually) in all investigated countries except Denmark. On the other hand, the incidence rate in Denmark (in 1995) was several-fold higher than in some of the other countries studied, e.g 5-fold higher than in Finland and 7-fold higher than in Lithuania (Richiardi *et al.* 2004). Also in France, Great Britain and most industrialized countries in North America and Oceania there is a clear trend towards an increased testicular cancer incidence (Moller 1998, Toledano *et al.* 2001, Huyghe *et al.* 2003, Walschaerts *et al.* 2008).

The incidence of hypospadias (ectopic urethral opening) is approximately 0.4 to 8.2 per 1000 male births and just as the testicular cancer incidence it is increasing in both North America and Europe (Paulozzi *et al.* 1997, Gallentine *et al.* 2001, Leung & Robson 2007). Cryptorchidism (undescended testes), another reproductive tract malformation, occurs in approximately 1-9% of newborn boys and appears to show the same trend over time as the reproductive parameters described above (Boisen *et al.* 2004). Chilvers and colleagues reported that the frequency of undescended testes in England and Wales was twice as high in 1981 as in 1962 (Chilvers *et al.* 1984). A similar result was obtained in a Danish study that compared the prevalence of cryptorchidism in the late 1990´s with 40 years earlier (Boisen *et al.* 2004).

It has been hypothesized that these reproductive disorders comprise one syndrome, the testicular dysgenesis syndrome (TDS), arising from a common underlying disturbance during fetal life (Skakkebaek *et al.* 2001, Sharpe & Skakkebaek 2008). A substantial amount of epidemiological, clinical and experimental data supports this hypothesis. Epidemiological studies have shown that a high incidence of testicular cancer is associated with a high frequency of hypospadias and cryptorchidism, and vice versa. For example, in Finland the rates of testicular cancer as well as hypospadias and cryptorchidism are 3-5-fold lower than in Denmark (Virtanen *et al.* 2001, Boisen *et al.* 2005, Virtanen *et al.* 2005). Clinical observations in patients with androgen insensitivity syndrome (AIS) also provide evidence for a common underlying entity. These patients experience not only disturbances in androgen-dependent processes such as testicular descent, but also an increased risk for developing testicular cancer (Savage & Lowe 1990). In fact, cryptorchidism is one of only a few well-established risk factors for testicular cancer (Dieckmann & Pichlmeier 2004).

In addition, it is possible to induce a TDS-like phenotype in laboratory animals by *in utero* exposure of male fetuses to high doses of the plasticizers di-*n*-butyl

phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP). This exposure regimen has been found to induce a high rate of disorders similar to the ones associated with human TDS, i.e hypospadias, cryptorchidism and infertility (Gray *et al.* 2000, Fisher *et al.* 2003).

The finding that environmental endocrine disruptors could interfere with male sexual differentiation and give rise to a TDS-like phenotype in animal models led to the theory that TDS in humans is induced not only by genetic defects but also by environmental factors. Genetic alterations such as 45X/46XY mosaicism and AR mutations give rise to severe forms of TDS, but for most cases of mild to moderate TDS the cause is still unknown, strengthening the hypothesis of environmental factors being involved (Skakkebaek *et al.* 2001, Yong *et al.* 2003, Joensen *et al.* 2008). Recent investigations do indicate a possible relationship between fetal EDC exposure and reproductive parameters in humans. Swan and colleagues found a significantly reduced anogenital distance (AGD), one of the most sensitive end-points for antiandrogenic activity, in human males that were exposed prenatally to phthalates (Swan *et al.* 2005). In addition, studies of human testis explants have revealed that fetal Leydig cell testosterone secretion can be affected at very low (biologically relevant) concentrations of EDCs (Fowler *et al.* 2007). On the other hand, as the understanding of male reproductive tract development has advanced, additional genetic causes have been identified. The over 300 AR mutations discovered so far are able to induce effects of varying severity, from mild TDS to complete feminization, and cryptorchidism has recently been associated with mutations in insulin-like factor 3 (INSL3) as well as its receptor leucine-rich repeat-containing G-protein-coupled receptor (LGR8) (Yong *et al.* 2003, Ferlin *et al.* 2006, Bogatcheva *et al.* 2007).

For this reason, it is important to investigate the interplay between environmental and genetic factors. It has been suggested that genetic polymorphisms may influence the susceptibility to EDCs and this possibility is important to further analyze.

Importantly, adverse effects of EDCs are not restricted to disturbances that occur as a consequence of prenatal exposure. It is known, mainly from studies of occupational exposure, that adult exposure to certain pesticides, heavy metals and organic solvents can severely affect reproductive functions and impair fertility in men (Lancranjan *et al.* 1975, Potashnik *et al.* 1978, Potashnik *et al.* 1979, Kelly 1988).

The following sections provide a more detailed description of the reproductive consequences of fetal, adolescent and adult exposure to each of the substances investigated in the experimental part of this thesis.

Cadmium (Cd)

Cd is a widespread environmental pollutant that occurs in nature at low concentrations. It was discovered in 1817, but the industrial use was minor until the beginning of the 20th century. Cd is used in polyvinyl chloride (PVC) products, colour pigments, alloys and rechargeable nickel-cadmium batteries. Additionally, Cd is used as an anti-corrosion agent and occurs in phosphate fertilizers. Since Cd occurs naturally in ores together with non-ferrous metals, zinc (Zn) and lead mining are important sources of environmental Cd pollution As a consequence of the implementation of a more stringent European Union environmental legislation (Directive 91/33/ECC), Cd usage within EU has decreased considerably since the 1990´s. However, the worldwide Cd consumption has increased dramatically during the 20th century (Jarup 2003, Nordberg *et al.* 2007).
Cd has a very long biological half-life, about 10-40 years, in the human body and is highly toxic (Nordberg *et al.* 1985). Cd may adversely affect several organs, such as the kidney, liver, placenta, bone and testis (Nordberg 1971, Goyer *et al.* 1994, Liu *et al.* 1998, Rikans & Yamano 2000, Piasek *et al.* 2001, Brzoska & Moniuszko-Jakoniuk 2004). Due to its high toxicity, long biological half-life and persistence in the environment Cd has gained an increased interest over the last few decades.

Cd exposure

Humans are generally exposed to Cd by two main routes, ingestion and inhalation. Cadmium chloride ($CdCl_2$) is the main form associated with ingestion, whereas Cd oxide (CdO) is the principle form associated with inhalation exposure (ATSDR 1999). In the general, non-smoking, population the largest source of Cd exposure is contaminated food. Most foods contain low concentrations of Cd (less than 0.1 mg/kg), but organ meat (kidney in particular) and shellfish may contain up to 1-2 mg/kg (WHO 2004). In heavily polluted areas, Cd concentrations in main food (e.g. rice) may exceed 2 mg/kg (Nordberg *et al.* 2002, Jin *et al.* 2004).
In 1989 the Food and Agriculture Organization/World Health Organization (FAO/WHO) Joint Expert Committee on Food Additives (JECFA) set the provisional tolerable weekly intake (PTWI) for Cd at 7 µg/kg body weight/week, which corresponds to 70 µg/day (WHO 1989). The same expert group kept the recommendation according to the most recent evaluation (WHO 2004). Apart from residents in heavily contaminated areas, the dietary Cd intake is below 70 µg/day for a majority of the general population. However, the intakes of Cd via foodstuff vary greatly between different countries. The

estimated daily dietary intake in Greece is significantly higher than in other European countries (50-70 vs. 10-30 µg) (Tsoumbaris & Tsoukali-Papadopoulou 1994, Nasreddine & Parent-Massin 2002) (WHO 2004). In Australia the dietary intake is estimated to be slightly higher than in most European countries, spanning from 20 to 30 µg/day, whereas in Japan the daily intake is approximately 20-70 µg (Satarug *et al.* 2002, Satarug *et al.* 2003, Watanabe *et al.* 2004),. Differences in dietary intakes of Cd are well reflected in the blood concentrations. Zhang and co-workers reported that the mean blood Cd concentration in Japanese women was 1.92 µg/l; a concentration several-fold higher than Cd concentrations recorded in German (0.44 µg/l) and American populations (0.77 µg/l) (Zhang *et al.* 1997, Becker *et al.* 2002, McKelvey *et al.* 2007).

Occupational exposure to Cd occurs primarily via the respiratory system, but may also to a smaller extent involve the gastrointestinal route (Jarup 2002). The smelting of non-ferrous metals and the production of Cd-containing particles raises Cd levels in the air. In the operations associated with these processes, Cd dust, and at temperatures high enough, Cd fume, arise (WHO 1992). In the 1940´s to 1960´s the air Cd concentrations often reached 1-5 mg/m^3 in occupational settings (Friberg 1950, Adams *et al.* 1969). Since then, significant improvements in occupational hygiene have been made and nowadays the concentrations in workplace environments are generally lower than 0.05 mg/m^3 (Jarup *et al.* 1998). However, during the last 10-15 years, several studies on occupational Cd exposure have shown that kidney dysfunction occurs at very low exposure levels (Mueller *et al.* 1992, Jarup & Elinder 1994, Jarup *et al.* 2000). This observation, together with the long biological half-life of Cd, is the reasons for occupational Cd exposure still being an important issue.

Another important source of Cd exposure is tobacco smoke, and a number of Swedish studies have shown that blood and kidney concentrations of Cd are significantly higher among smokers than non-smokers (Elinder *et al.* 1983a, Nilsson *et al.* 1995, Barregard *et al.* 1999, Nordberg *et al.* 2000). For example, Elinder and and colleagues found that ~ 90% of investigated smokers had blood Cd concentrations above 0.6 µg/l, whereas ~ 90% of non-smokers showed concentrations lower than 0.6 µg/l (Elinder *et al.* 1983a). Consistently, a more recent study reported the mean Cd concentrations in smokers and non-smokers were 1.1 µg/l and 0.28 µg/l, respectively (Becker *et al.* 2002).

One cigarette contains approximately 1-2.5 µg Cd, of which 10-20% is inhaled during smoking (Menden *et al.* 1972, Friberg 1974, Elinder *et al.* 1983b, Saldivar *et al.* 1991, Kalcher *et al.* 1993). During the burning of cigarettes highly bioavailable CdO is generated and approximately 30-40% of the inhaled CdO passes through the pulmonary epithelium into systemic circulation,

whereas another 10% is deposited in lung tissues (ATSDR 1999, Zalups & Ahmad 2003, Satarug & Moore 2004). Based on these data it can be estimated that a person smoking one package of cigarettes a day will absorb about 1.5-4.5 µg Cd. Importantly, inhalation of tobacco smoke raises the blood Cd concentrations not only in active smokers but also in passive ones (Willers *et al.* 1992, Shaham *et al.* 1996).
Although the Cd intake for most populations does not exceed PTWI there is good reason for precaution. Recent studies clearly indicate that the current PTWI is not restrictive enough to protect the general population. For example, Hellström and collaborators found Cd-induced kidney dysfunction after dietary exposure levels that were well within PTWI (Hellstrom *et al.* 2001).

Cd toxicokinetics

As mentioned above, the main routes of human Cd exposure are inhalation and ingestion. Inhalation exposure occurs in the form of aerosols. A significant proportion of inhaled Cd ends up in the gastrointestinal tract, since large Cd-containing particles of limited solubility are translocated by mucociliary transport (Moore *et al.* 1973, Adamsson *et al.* 1979). More finely dispersed Cd aerosols, on the other hand, are efficiently absorbed. Whereas the absorption of Cd in the lungs is about 50%, the gastrointestinal uptake is much less efficient and only a few percent is absorbed (Jarup *et al.* 1998).
The gastrointestinal uptake of Cd occurs mainly in the duodenum and early jejunum (Andersen *et al.* 1994). The chemical form of Cd present in the intestine greatly influences the absorption as well as the subsequent transport to target organs (IARC 1992). Depending on the dietary source of Cd, there are several different forms of Cd that can be presented to the enterocytes of the intestinal mucosa (Zalups & Ahmad 2003). The absorption rate is also determined by factors such as animal species, nutritional status and stage of development. The nutritional iron status appears to be particularly important and iron deficiency can lead to significantly increased Cd absorption (Flanagan *et al.* 1978, Akesson *et al.* 2002, WHO 2004).
Cd bound to metallothionein (Cd-MT) can cross the epithelium in intact form and enter into the capillaries of lamina propria to be delivered into portal circulation (Cherian 1979). The major mechanism for Cd uptake in enteroytes was believed to rely on divalent metal transporter 1 (DMT1), a transmembrane transport protein capable of transporting iron (Fe) and a number of other divalent cations (Conrad & Umbreit 2000, Zalups & Ahmad 2003). However, a recent study of DMT1-dysfunctional mice has revealed the existence of another, yet unidentified, pathway for intestinal Cd absorption (Suzuki *et al.* 2008). The basolateral transport of Cd is mediated by metal transporter protein

1 (MTP1) (Ryu *et al.* 2004). Additionally, high Cd concentrations can damage the plasma membrane, resulting in the release of Cd to the capillaries (Zalups & Ahmad 2003). Once inside the enterocyte, Cd can induce the transcription of metallothionein (MT) 1 and 2. The binding of Cd to MT-1 and MT-2 leads to retention of Cd in the intestinal mucosa and subsequently a lowered amount of Cd reaching the target organs (Ouellette *et al.* 1982, Lehman & Klaassen 1986).

After its entry into systemic circulation Cd binds primarily to albumin, which transports Cd to the epithelial cells in target organs (Nordberg & Nordberg 1987). Other transport molecules for Cd in blood plasma are MT, transferrin and possibly the low-molecular-weight thiols glutathione (GSH) and cysteine (Cys) (Nordberg & Nordberg 1975, Chan *et al.* 1993, De Smet *et al.* 2001, Zalups & Ahmad 2003). Cd bound to albumin is mainly taken up by the liver, whereas Cd bound to MT is preferentially distributed to the kidney (Nordberg & Nordberg 1975, Groten *et al.* 1991).

Regardless of exposure route, the liver is by far the primary organ that takes up the largest quantity of Cd during the initial hours after exposure (Kjellstrom & Nordberg 1978). A large proportion of Cd absorbed in the intestine is delivered first to the liver via portal circulation. In the liver Cd is taken up by hepatocytes from the sinusoidal capillaries. The subsequent up-regulation of, and binding to, MT-1 and MT-2 causes retention of Cd in the liver and thereby constitutes an important detoxifying mechanism (Coyle *et al.* 2002). However, when hepatocytes get overloaded with Cd the protective mechanisms are overwhelmed and oxidative stress, lipid peroxidation and finally cell death arise (Stohs *et al.* 2000, Kim *et al.* 2003). As a consequence, Cd bound to MT is released into hepatic circulation from apoptotic/necrotic hepatocytes (Zalups & Ahmad 2003). Importantly, some of the Cd taken up by hepatocytes is secreted into the biliary canaliculi and subsequently into the common bile duct and the duodenum. In this way a portion of Cd can be excreted via the feces.

As mentioned earlier the kidney is sensitive to Cd exposure and renal dysfunction can arise at exposure levels lower than PTWI (Hellstrom *et al.* 2001). Due to its small size, Cd bound to MT is filtered freely at the glomerulus and Cd-MT is the predominant form present in the luminal compartment of the nephron (Jarup *et al.* 1998). Cd-albumin is not as efficiently filtered through the glomerular membrane and hence only a small percentage of Cd bound to albumin in plasma passes into the proximal tubule lumen. Both Cd-MT and Cd-albumin is taken up by tubular cells through endocytosis (Choi *et al.* 1999, Erfurt *et al.* 2003). Similar to its actions in the liver, Cd induces the transcription of MT-1 and MT-2 genes, and at high levels it causes oxidative stress, lipid peroxidation and interference with CaMg-ATPase in basolateral membranes (Jarup *et al.* 1998, Leffler *et al.* 2000).

Reproductive effects of Cd: insights from animal models

Adverse reproductive effects of Cd exposure in animals were reported as early as the mid 1950´s (Parizek & Zahor 1956). The testis is particularly sensitive, but exposure to Cd alters also ovarian, adrenal and pituitary functions (Piasek & Laskey 1994, Ricard *et al.* 1998, King *et al.* 1999, Piasek & Laskey 1999). In addition, Cd is a potent teratogen (Fernandez *et al.* 2003). Cd administered during gestation gives rise to profound teratogenic effects in a number of species, including rodents and amphibians (Chernoff 1973, Sunderman *et al.* 1992). The nature of the changes induced is dependent on the dose as well as the stage of embryogenesis and the species studied (Thompson & Bannigan 2008). In *Xenopus laevis* Cd treatment causes gut malformations and heart lesions, whereas in rodents it affects neural tube closure and limb development (Webster & Messerle 1980, Sunderman *et al.* 1992).

At lower doses, *in utero* Cd exposure influences reproductive parameters. Johnson and collaborators found that Cd treatment during gestation caused estrogen-like effects in the female offspring (Johnson *et al.* 2003). The authors reported that *in utero* exposure to Cd (0.5 and 5 µg/kg bw) advanced vaginal opening and increased the epithelial area and number of terminal end buds in the mammary gland. Cd is known to bind ERs and activate ER-dependent gene transcription *in vitro*, and this mechanism is likely to underlie the estrogenic activity *in vivo* (Stoica *et al.* 2000, Wilson *et al.* 2004a). Consistent with this theory, Johnsson and colleagues observed that the co-treatment with the antiestrogen ICI-182,780 blocked the *in vivo* estrogenic effects of Cd. Perinatal exposure to low doses of Cd has also been found to affect the expression of ERα, ERβ and PR in the brains of male and female mice (Ishitobi *et al.* 2007).

Cd has been found to accumulate in the ovary and in the adult female animal it inhibits ovulation and affects ovarian steroidogenesis (Varga *et al.* 1993, Piasek & Laskey 1994, 1999). In addition, Cd has been reported to exhibit estrogenic activity, manifested by increased uterine weight and promoted development of mammary glands, also in the adult (Johnson *et al.* 2003). Cd has been found to affect gonadal steroidogenesis in nonpregnant as well as pregnant animals. Piasek and Laskey described the effects of subcutaneous Cd injections (3 or 5 mg/kg bw) on steroidogenesis in cycling and pregnant rats (Piasek & Laskey 1994). Both doses significantly reduced progesterone and estradiol synthesis, with the most pronounced effects seen on estradiol production in proestrus or early pregnancy. Mechanistic studies have revealed that Cd inhibits progesterone synthesis in granulosa cells by down-regulation of StAR and p450scc (Zhang & Jia 2007). The same article reported that co-treatment with 8-bromo-cAMP blocked the decline in progesterone secretion,

indicating that Cd exerts its action by interfering with cAMP synthesis and/or signalling, which in turn leads to a reduced expression of StAR and p450scc.

In the male gonad, Cd can cause testosterone suppression, failure of spermiation, reduced sperm motility, increased incidence of Leydig cell tumors, and at high doses testicular damage (Nordberg 1971, Laskey *et al.* 1984, Bomhard *et al.* 1987, Waalkes *et al.* 1988, Laskey & Phelps 1991, Hew *et al.* 1993, King *et al.* 1998, Liu *et al.* 2001, Yang *et al.* 2003, El-Demerdash *et al.* 2004). Cd is localized mainly in interstitial blood vessels and to some extent in Leydig cells, whereas only very low/undetectable concentrations are found other testicular cell types (Nordberg 1972, Danielsson *et al.* 1984, Bench *et al.* 1999). Although only 1-2% of an administered Cd dose is taken up by the testis, it is particularly sensitive (Gunn *et al.* 1968). Cd up-regulates the MT-1 and MT-2 transcription also in the testis but lacks the ability to induce MT translation, which has been suggested as the underlying mechanism (Ren *et al.* 2003b, 2003a). In addition, it is likely that the major Cd-binding protein in the testis is not MT, but a different metal-binding protein named testicular metal-binding protein 3 (TMBP-3) (Waalkes *et al.* 1984).

Exposure to Cd, *in vitro* as well as *in vivo*, reduces testosterone synthesis. The inhibitory effect of Cd on steroidogenesis is detected at doses/concentrations that do not affect Leydig cell viability or induce testicular necrosis, indicating a specific disruptive mechanism (Laskey *et al.* 1984, Laskey & Phelps 1991). Although Leydig cell testosterone synthesis is very sensitive to Cd exposure, Leydig cells seem to be much more resistant than other testicular cell types to the general toxicity induced by Cd. At Cd exposure levels associated with apoptotic or necrotic events in spermatocytes and Sertoli cells no degenerative effects are detected in the Leydig cells (Zhou *et al.* 1999, Lymberopoulos *et al.* 2000). Recent findings indicate that more subtle changes within Leydig cells could trigger the apoptosis of other testicular cells. Ozawa and co-workers found that heme oxygenase-1 (HO-1) derived from Leydig cells induced apoptosis of premeiotic germ cells and testosterone is well-known germ cell survival factor (Erkkila *et al.* 1997, Ozawa *et al.* 2002).

Although the detrimental effect of Cd on testicular steroiodogenesis has been known for a long time, the underlying mechanisms largely remain to be discovered. Laskey and Phelps concluded from *in vitro* experiments that Cd was able to block human chorionic gonadotropin (hCG)-stimulated testosterone synthesis via inhibitory site(s) of action subsequent to the LH receptor and cAMP production, but prior to p450scc (Laskey & Phelps 1991). In contrast, it is known from steroidogenic tissues other than the testis that Cd can reduce cAMP synthesis. Mgbonyebi and colleagues discovered that Cd could inhibit unstimulated as well as adrenocorticotropic hormone (ACTH)-stimulated cAMP secretion in mouse adrenal cells (Mgbonyebi *et al.* 1994).

Apart from direct effects on gonadal steroidogenesis, Cd exposure may influence pituitary function, either by affecting the secretion of pituitary hormones or changing the pituitary responsiveness to gonadotropin releasing hormone (GnRH). Lafuente and colleagues discovered that Cd altered the pituitary secretion of gonadotropins as well as prolactin in male rats (Lafuente *et al.* 2003). Interestingly, Cd had a dose-dependent biphasic effect on prolactin secretion. The lowest dose (5 ppm) stimulated the secretion of prolactin, whereas higher doses were inhibitory. Poliandri and colleagues found a rapid decline in prolactin secretion in primary pituitary cells exposed to Cd, indicating a direct effect on the pituitary (Poliandri *et al.* 2003). However, Cd may also alter the dopaminergic control of prolactin secretion, by reducing the dopamine content in the median eminence (Lafuente *et al.* 2005). In addition, Cd alters the responsiveness of the pituitary to GnRH. Szczerbik and colleagues injected fish with a GnRH analogue and discovered that gonadotropin secretion was not induced to the same extent in Cd-treated animals as in controls (Szczerbik *et al.* 2006).
It has been known for almost 50 years that Zn can protect testicular tissue against Cd-induced effects (Parizek & Zahor 1956, Webb 1972). Although not extensively studied in the testis, findings from other tissues have clarified the underlying mechanisms. Zn pre-treatment induces metallothionein synthesis, prevents oxidative stress and alters Cd toxicokinetics; all of which possibly contribute to the protective effect (Waalkes & Perantoni 1988, Chan & Cherian 1992, Powell 2000). However, as suggested by Khan and colleagues, there are considerable tissue differences (Khan *et al.* 1991).

Possible reproductive effects in humans

The possible effects of Cd exposure on human development and reproduction have been investigated in a number of studies. Several reports have described an association between maternal Cd exposure and reduced birth weight of the child. Huel and co-workers found an inverse relationship between Cd content in the hair of newborn children and their birth weight (Huel *et al.* 1981). Consistent with this, increased Cd concentrations have been detected in cord blood of infants with low birth weight (Salpietro *et al.* 2002). Nishijo and colleagues found that Cd exposure reduced the gestational age, a finding that may explain previous observations of decreased birth weight in Cd-exposed children (Nishijo *et al.* 2002). Later, the same researcher found a negative correlation between maternal blood Cd and infant height that was not due to early delivery (Nishijo *et al.* 2004).
A few studies have analyzed the influence of Cd exposure on male reproductive parameters, but at present there is no solid evidence that Cd is a

contributing factor to the decline in male reproductive health. Keck and co-workers found no correlation between Cd concentrations and semen parameters or fertility status, which was in accordance with previous studies (Saaranen *et al.* 1989, Keck *et al.* 1995). Menke and colleagues analyzed hormone concentration and reported that there were no association between urinary Cd levels and serum sex hormone concentrations in an American population (Menke *et al.* 2008). However, other studies have detected alterations in reproductive hormone levels, in the general population as well as in particularly exposed populations. In Croatian males, without occupational exposure, a significant association between blood Cd and increased serum testosterone, estradiol and follicle stimulating hormone (FSH) concentrations was discovered (Jurasovic *et al.* 2004). Consistently, Zeng and collaborators found a dose-response correlation between urinary Cd and serum testosterone concentrations in a population living near a smelter, whereas another study revealed that occupationally exposed men (n=2) had, apart from ~ 7-fold higher Cd concentrations in seminal plasma, significantly elevated serum FSH levels (Keck *et al.* 1995, Zeng *et al.* 2004).
Cd is suspected to alter steroid levels also in women. Piasek and colleagues noted that placentas of smokers contained only half as much progesterone but twice the Cd content as non-smokers (Piasek *et al.* 2002). In addition, a recent report shows that Cd exposure is significantly associated with increased risk to develop endometrial cancer; an estrogen-dependent neoplasm (Akesson *et al.* 2008). This finding is consistent with the estrogenic activity associated with Cd exposure in animal models, but needs further verification since it was based on estimated dietary intake rather than actual exposure.

Phthalates

Phthalates are a group of industrial chemicals that are extensively used in a wide range of consumer products, including cosmetics, PVC floors, toys, car interiors and medical devices. They are manufactured in a very large scale, and in Western Europe alone the annual production of phthalates is about one million tons (Jaakkola & Knight 2008). Phthalates have a common chemical structure consisting of an aromatic ring and two, usually aliphatic, side chains of varying length. Low molecular weight phthalates (i.e short side chains), such as diethyl phthalate (DEP) and dimethyl phthalate (DMP) are used in cosmetic formulations. Phthalates of high or intermediate molecular weight, on the other hand, are used as plasticizers in numerous plastic products. Di-(2-ethylhexyl) phthalate (DEHP) and di-iso-nonyl phthalate (DINP) are examples

of high molecular weight phthalates, whereas di-*n*-butyl phthalate (DBP) and benzyl butyl phthalate (BBP) are of intermediate weight. Since phthalates are not chemically bound to the plastic polymer they are continuously released into indoor air, atmosphere, food products or directly into body fluids from medical devices (Koch *et al.* 2006).
Phthalates have a low acute toxicity (LD 50: 1-30 g/kg/bw) and lack mutagenicity and/or genotoxicity. Still, several high and intermediate weight phthalates, including the ones mentioned above, have been identified as endocrine disruptors capable of altering male sexual differentiation in laboratory animals. In contrast, the short-branched ones appear to lack such properties (Foster *et al.* 2000, Gray *et al.* 2000).

Phthalate exposure

In the general population, ingestion and inhalation are the primary routes of exposure to phthalates with high molecular weight (Silva *et al.* 2003). For phthalates with shorter side chains (e.g DEP) dermal absorption may be an important exposure route (Elsisi *et al.* 1989, Hauser & Calafat 2005). Due to the leakage from medical devices, patients that undergo intensive care may be exposed to significantly higher levels of phthalates than the general population. Infants are particularly exposed and plasma levels of mono-(2-ethylhexyl) phthalate (MEHP), the primary metabolite of DEHP, as high as 15.1 µg/ml (54 µM) have been recorded during neonatal exchange transfusions (Sjoberg *et al.* 1985).
Assessment of phthalate exposure can be based on ambient monitoring data or analysis of specific metabolites in the urine. Using the previous method, the daily intake of DBP and DEHP has been estimated to 2-10 µg/kg/day and up to 30 µg/kg/day, respectively. Children, which suck on toys and other daily life products, are generally more exposed to phthalates than adults. In a Canadian study, using the same methodology, toddlers were estimated to have a more than 3 times higher DEHP exposure than adults (19 µg/kg/day vs. 6 µg/kg/day) (Meek & Chan 1994).
As a consequence of ubiquitous exposure, concentrations of phthalate metabolites are usually high enough to be detected in human urine, enabling exposure assessments based on such data. For low molecular weight phthalates, urinary concentration of their respective primary monoester metabolite corresponds well with exposure to the parent compound (Silva *et al.* 2007). In contrast, the secondary oxidized metabolites of DEHP and DINP better reflect the exposure level than MEHP and and mono-iso-nonyl phthalate (MINP) (McKee *et al.* 2002, Heudorf *et al.* 2007, Wittassek & Angerer 2008). After oral exposure in humans, most of the DEHP dose is excreted in the urine

as secondary oxidized metabolites and only a few percent as MEHP (Koch *et al.* 2006). In addition, different elimination half-lives of different oxidized metabolites make them suitable biomarkers for both chronic and short-term DEHP exposure (Wittassek & Angerer 2008). By incorporating excretion data for several secondary oxidized metabolites the method also takes into account metabolic differences between individuals (Koch *et al.* 2006). As described below, children have a higher proportion of secondary oxidized DEHP metabolites than adults and such differences could lead to erroneous estimations if only the primary monoester was considered (Wittassek & Angerer 2008).

Analysis of urine has a number of advantages over ambient monitoring data. The most important, from a toxicological point of view, is that urine analyses provide concentration data for specific metabolites. Such data is of great value since it is the primary monoester metabolites and possibly the secondary metabolites (rather than the parent phthalates) that are considered to cause endocrine disruption (Davis *et al.* 1994, Mylchreest *et al.* 2000, Stroheker *et al.* 2005, Meeker *et al.* 2007). Importantly, however, since metabolite patterns may differ markedly between urine and serum, exposure assessments should be based solely on data from one of these matrices (Kato *et al.* 2004).

During recent years DINP has replaced DEHP in many products, a fact that is well reflected in the pattern of excreted phthalate metabolites. Based on the urinary concentration of primary and secondary metabolites, Wittassek and colleagues estimated that the daily intake of DEHP was reduced with approximately 40% during 1988-2003, whereas the daily intake of DINP was increased by 100% during the same time period. In addition, the authors observed a significant decline in DBP exposure (Wittassek *et al.* 2007).

Prenatal and neonatal exposure

Since phthalates have been detected in breast milk as well as in cord blood of newborns, exposure to phthalates occur during the fetal as well as the neonatal period in humans (Latini *et al.* 2003, Hogberg *et al.* 2008).

The primary metabolites of low and intermediate molecular weight phthalates, e.g monomethyl phthalate (MMP) and mono-*n*-butyl phthalate (MBP), have been found to cross the placenta in rats as well as humans (Fennell *et al.* 2004, Mose *et al.* 2007b). Whether MEHP has the ability or not to cross the placenta needs further investigations (Lashley *et al.* 2004, Mose *et al.* 2007b). Latini and collaborators detected DEHP and MEHP in a majority (77%) of cord serum samples from newborn children. On the other hand, results from a human placenta perfusion system indicate no placental transfer of MEHP (Mose *et al.* 2007b).

Breast milk, in contrast to urine, contains mainly unmetabolized phthalates and primary monoester metabolites. Two recent studies, one American and one Swedish, show that unmetabolized DEHP is the predominate phthalate in milk (0.45-305 ng/ml in the Swedish study) (Zhu *et al.* 2006b, Hogberg *et al.* 2008). Both reports detected DBP as the second most abundant compound, but only low concentrations of DEP/ monoethyl phthalate (MEP). The Swedish report also established that the breast milk contained MEHP (0.49-6.5 ng/ml), whereas concentrations of the oxidized metabolites mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP) and mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) were undetectable (Hogberg *et al.* 2008). In addition, the primary monoester phthalates are usually excreted in their free (unconjugated) form in breast milk (Calafat *et al.* 2004). Hence, nursing infants are exposed primarily to the bioactive metabolites or their precursors.

Phthalate metabolism

Following oral ingestion, the diester phthalates are rapidly hydrolyzed (phase I biotransformation) by intestinal lipases to their corresponding monoester phthalate, e.g DEHP to MEHP, which is easily absorbed from the gut (Kluwe 1982, Ljungvall *et al.* 2004). Upon inhalation or dermal exposure, phthalates are absorbed as the parent compound and thereafter metabolized to the monoester form (Shea 2003, Mose *et al.* 2007a). Since the rate of dermal absorption decreases with increasing length of the side chains, DEHP is significantly less well absorbed than DEP and DBP (Elsisi *et al.* 1989, Janjua *et al.* 2008). For this reason, dermal exposure may be an important route of exposure for DEP and DBP, but hardly for DEHP and DINP.
The monoester phthalates can be either excreted unchanged or undergo further biotransformations, i.e hydroxylation and oxidation followed by phase II glucuronidation (Figure 1) (Frederiksen *et al.* 2007). Monoester forms of low molecular weight phthalates are relatively hydrophilic and excreted mainly in their free form, whereas MEHP, MBP and MINP undergo (to a large extent) further biotransformations (Silva *et al.* 2003, Mose *et al.* 2007a). MBP is excreted primarily as its glucuronide conjugate, whereas the long chain phthalates have a more complex metabolic pattern involving several secondary metabolites, which are excreted either conjugated or unconjugated (Wittassek & Angerer 2008).
Diester phthalates usually become more bioactive after hydrolysis to the monoester metabolites and these, rather than the parent compounds, are considered to be the active agents in testicular as well as ovarian toxicity (Sjoberg *et al.* 1986, Davis *et al.* 1994, Mylchreest *et al.* 2000).

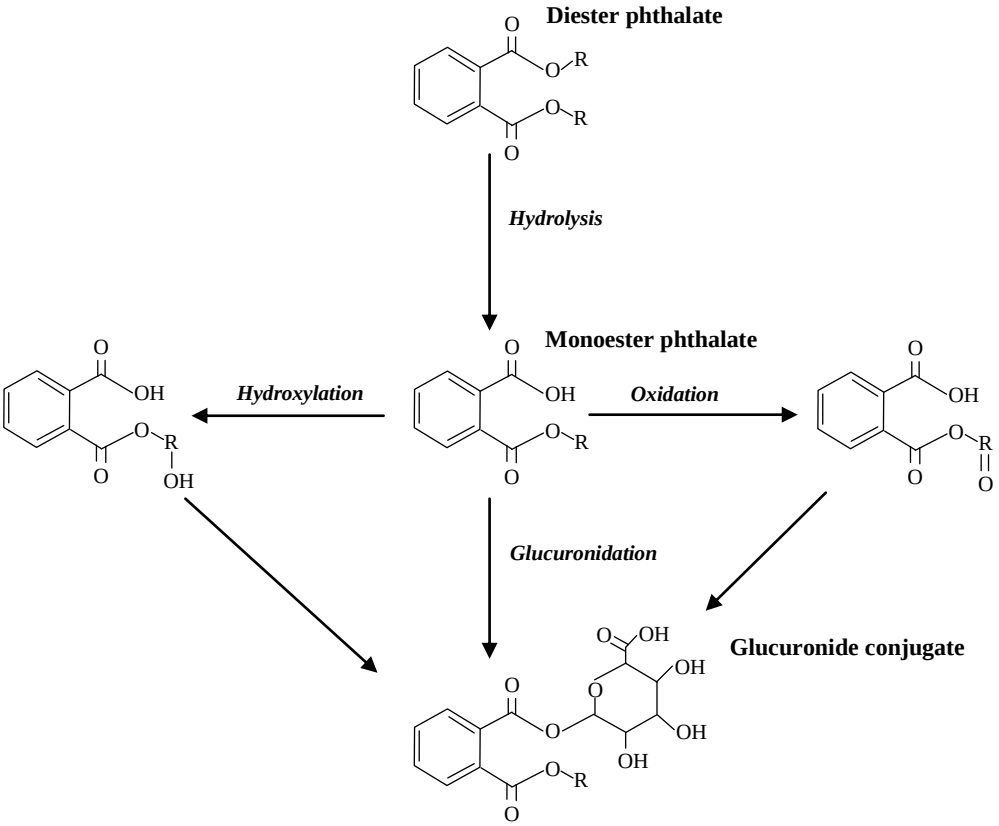

Figure 1. General metabolic pathway for phthalates (Modified from Frederiksen *et al.* 2007).

Importantly, phthalate metabolism differs between species as well as within a species. Inter-species differences appear to be more pronounced for high molecular weight phthalates. MBP is the major urinary metabolite of DBP in both rats and humans, whereas the metabolism of DEHP differs markedly between species (Tanaka *et al.* 1978, Silva *et al.* 2007). For example, Ito and colleagues discovered drastic differences in lipase activity and thus the hydrolysis of DEHP to MEHP, between rodents and marmosets (Ito *et al.* 2005). The authors detected species differences also in the activity of enzymes involved in oxidation and glucuronidation of DEHP metabolites (e.g alcohol

dehydrogenase and UDP-glucuronyl transferase), but these were less pronounced. As proposed already in 1986 by Rhodes and collaborators, such characteristic differences in the metabolism may explain why primates are less sensitive to DEHP exposure than rodents (Rhodes *et al.* 1986). In other cases, two strains of the same species show different sensitivity to DEHP exposure. Wistar rats are known to be much more susceptible to phthalate-induced cryptorchidism than Sprague-Dawley rats (Wilson *et al.* 2007). However, whether this is caused by metabolic differences, varying sensitivity to one/several metabolites or differences in the regulation of reproductive tract development is not yet known.
The age of an individual is also an important factor influencing the metabolism of phthalates. A German study revealed that the relative proportion of MEHP to its secondary (oxidized) metabolites differed between children of different ages (Becker *et al.* 2004). In accordance with this finding, the same researchers later discovered that children (6-7 years of age) excreted approximately four times more oxidized metabolites compared to MEHP than adults (Wittassek & Angerer 2008).

Reproductive effects of phthalates: insights from animal models

Diester phthalates as well as their active monoester metabolites exhibit no or low affinity for steroid hormone receptors (Parks *et al.* 2000). DINP, DEHP, DBP and their corresponding monoester metabolites do not bind to the AR (Mylchreest *et al.* 1999, Parks *et al.* 2000, Kruger *et al.* 2008). Still, these compounds cause reproductive and developmental effects (e.g hypospadias and cryptorchidism) in male laboratory animals that are very similar to the ones caused by exposure to known AR antagonists such as flutamide (Mylchreest *et al.* 1999). This TDS-like phenotype, which occurs as a consequence of *in utero* exposure to relatively high doses of phthalates, is probably the most recognized reproductive effect associated with phthalate exposure. However, phthalates also affect female reproductive functions and at low doses some of them give rise to more subtle effects in both sexes, such as advanced pubertal onset (Ma *et al.* 2006, Ge *et al.* 2007).
The fact that phthalates lack affinity for AR made some researcher to hypothesize that the reported developmental disturbances should be attributed to the their estrogenic potential (Jobling *et al.* 1995). DINP, DEHP and DBP have been identified as weak activators of ERα dependent gene transcription *in vitro* (Harris *et al.* 1997, Takeuchi *et al.* 2005). However, the estrogenic activity is most probably not the mechanism underlying the feminizing effects of phthalates since they lack estrogenic properties *in vivo*. For example, DBP at doses that alter male reproductive tract development, i.e 200-1000

mg/kg/day, failed to increase uterine weight in juvenile rats and did not accelerate vaginal opening (Gray *et al.* 2005). Instead, recent studies have revealed that phthalates act by mechanisms that do not involve either AR or ER.

Thanks to the work of several research groups it is now well established that a number of high and intermediate molecular weight phthalates may disturb the development of the reproductive tract and induce a TDS-like phenotype in male laboratory animals. About a decade ago it was discovered that gestational and lactational exposure to DBP affected reproductive tract development in male rats (Mylchreest *et al.* 1998). At 500 and 750 mg/kg/day DBP, the authors detected a decreased AGD at birth and a high incidence of hypospadias and cryptorchidism. Other alterations found in the male offspring were absent/underdeveloped epididymis and germ cell loss. Shortly thereafter, Gray and co-workers reported similar effects after exposure to DEHP (Gray *et al.* 1999). A subsequent study by the same authors analyzed several phthalates and revealed that perinatal exposure, from gestational day 14 to postnatal day (PND) 3, to 750 mg/kg/day of DEHP, BBP or DINP resulted in reproductive malformations and induced female-like areolas/nipples in the male offspring (Gray *et al.* 2000). DINP was found to be less potent than DEHP and BBP, whereas the short-branched phthalates DEP and DMP did not influence male sexual differentiation. Since then a number of studies have confirmed these findings and provided insight into the underlying mechanisms (e.g Fisher *et al.* 2003, Wilson *et al.* 2004b, Andrade *et al.* 2006b, Mahood *et al.* 2007).

The phenotype induced by phthalates is characteristic of a disturbance in androgen-regulated development and typically comprises malformations of the vas deferens, epididymis, seminal vesicles and prostate, in combination with cryptorchidism, hypospadias and reduced AGD (Gray *et al.* 1999). Hence, for phthalates to give rise to these adverse reproductive effects exposure must occur during the period of sexual differentiation late in gestation.

Importantly, phthalates cause effects similar to, as well as different from, the ones seen after exposure to AR antagonists. Both phthalates and AR antagonists, such as flutamide and vinclozolin, induce hypospadias, cryptorchidism and a reduced AGD (Mylchreest *et al.* 1999, Shono *et al.* 2004). However, whereas phthalates significantly reduce fetal testis testosterone production, vinclozolin and flutamide act solely at the level of AR (in androgen-dependent tissues) and do not influence the testosterone synthesis (Mylchreest *et al.* 2002, Wilson *et al.* 2004b). Another difference between phthalates and vinclozolin is that the former down-regulates testicular INSL3 expression (Wilson *et al.* 2004b, Lague & Tremblay 2008). INSL3 and testosterone are important regulators of different phases in testicular descent. The transabdominal phase is under control of INSL3, whereas testosterone

regulates the inguinoscrotal phase (Virtanen *et al.* 2007). Consistent with this, phthalate exposure or targeted disruption of the INSL3 gene causes primarily intraabdominal cryptorchidism, whereas flutamide induces mainly inguinal cryptorchidism (Mylchreest *et al.* 1999, Zimmermann *et al.* 1999). Whether or not the phthalate-induced reduction in INSL3 expression is secondary to decreased testosterone concentrations is not yet clarified. Lague and Tremblay found that testosterone stimulated INSL3 expression *in vitro* in Leydig cells, by a mechanism that requires AR, but this regulatory mechanism remains to be verified *in vivo* (McKinnell *et al.* 2005, Lague & Tremblay 2008).

Another effect associated with fetal phthalate exposure is the occurrence of focal areas of testicular dysgenesis (postnatally) comprising malformed seminiferous tubules and intratubular Leydig cells (Fisher *et al.* 2003). Based on analyses of fetal testes from DBP-exposed rats, Mahood and co-workers proposed that this postnatal phenotype is caused by abnormal Leydig cell aggregation in the fetal testis (Mahood *et al.* 2005). In their study, 500 mg/kg/day DBP induced the formation of large Leydig cell aggregates that were located centrally in the fetal testis and contained trapped Sertoli cells. They hypothesized that as Sertoli cells attempt to form seminiferous cords early in postnatal life the process is disturbed by this aggregation, resulting in malformed seminiferous tubules that contain Leydig cells. Recently, up-regulation of leukemia inhibitory factor (LIF) was identified as the likely mechanism underlying phthalate-induced fetal Leydig cell aggregation. Lin and colleagues discovered not only that *in utero* exposure to DEHP increased testicular LIF mRNA expression, but also that LIF treatment caused Leydig cell aggregation *in vitro*, in a concentration-dependent manner (Lin *et al.* 2008).

Apart from the effects described above, phthalates influence the onset of puberty. High-dose gestational and lactational exposure to DEHP, BBP or DBP markedly delays the onset of puberty, as measured by the age of preputial separation, in male rats (Gray *et al.* 1999, Mylchreest *et al.* 1999, Tyl *et al.* 2004, Andrade *et al.* 2006a). In contrast, postnatal exposure to low doses of DEHP advances pubertal onset in rats of both sexes. Ge and co-workers found that male rats administered 10 mg/kg/day DEHP (from PND 21 to 48) completed preputial separation significantly earlier than control animals (39.7 ± 0.1 days vs. 41.5 ± 0.1 days) (Ge *et al.* 2007). Ma and colleagues reported similar effects after inhalation exposure to DEHP in female rats (Ma *et al.* 2006). They noted that exposure to 5 mg/m^3 DEHP (from PND 22 to 41) advanced the age at vaginal opening as well as the first estrus. Hence, in contrast to *in utero* exposure, which has very limited effects on the female fetus, postnatal exposure to phthalates may affect both sexes. This is supported by the finding that DEHP, although at very high doses (2 g/kg/day), prolongs

estrus cycles and disturbs ovulation in adult female rats (Lovekamp-Swan & Davis 2003).

Possible reproductive effects in humans

In recent years a number of reports have been published suggesting a possible association between phthalate exposure and reproductive or developmental effects in humans. In a Puerto Rican study, Colon and colleagues discovered significantly elevated serum concentrations of DEP, DMP, DBP, DEHP and MEHP in 68% of girls with premature breast development (Colon *et al.* 2000). Swan and co-workers reported that AGDs were reduced in male infants prenatally exposed to phthalates, whereas an Indian study revealed that women suffering from endometriosis had significantly higher concentrations of DEHP, DBP and BBP than unaffected women (Swan *et al.* 2005, Reddy *et al.* 2006). Phthalates have also been suggested to shorten the duration of pregnancy, since MEHP-positive newborns show significantly lower gestational age than MEHP-negative controls, and induce sperm DNA damage (Latini *et al.* 2003, Hauser *et al.* 2007)
In addition, two studies from 2007 describe an association between phthalate exposure and altered thyroid hormone levels in men as well as women. Huang and collaborators reported an inverse relationship between urinary MBP and serum thyroxine (T_4) and free T_4 in pregnant women (Huang *et al.* 2007). In accordance with this finding, a negative correlation between urinary MEHP and serum total triiodothyronine (T_3) and free T_4 was found in adult men (Meeker *et al.* 2007).

Phytoestrogens

Since ancient times it has been known that certain plants can influence fertility, but it was not until the 1940´s that research was initiated to identify the active components. In 1946 Bennets and co-workers reported that subterranean clover (*Trifolium subterraneum*) caused infertility in pasture-grazing sheep in Western Australia, a phenomenon later attributed to intrinsic plant compounds termed "phytoestrogens" (Bennets *et al.* 1946). Phytoestrogens are biologically active, nonsteroidal, plant substances that mimic the effects of endogenous estrogens. They may do so by binding to ERα/ERβ, induce estrogen-mediated gene transcription and thereby stimulate growth of the female genital tract or change the amount of circulation endogenous hormones (Kurzer & Xu 1997). Phytoestrogens can be divided into four classes: flavonoids, lignans,

phytosterols and terpenoids (Figure 2) (Vajda & Norris 2006). The flavonoid class consists of several subgroups, of which isoflavones (e.g genistein, daidzein, biochanin A, formononetin), isoflavans (e.g equol) and coumestans (e.g coumestrol) are the most thoroughly studied with regard to estrogenic activity. An additional class, consisting of mycotoxins with estrogenic properties (mycoestrogens), is sometimes included in the term phytoestrogens, but mycoestrogens are in fact metabolites of fungal species and not intrinsic plant compounds (Kurzer & Xu 1997).

Although originally identified as compounds with estrogenic activities, phytoestrogens are now known to influence also androgen and thyroid hormone signalling (Chang & Doerge 2000, Doerge & Sheehan 2002, Stroheker *et al.* 2004, Lazarevic *et al.* 2008). In addition, some phytoestrogens act both as agonists and antagonists of estrogen action, depending on dose and target tissue. For this reason, some researchers use the more inclusive term endocrine-active phytochemicals (EAPs).

Since the studies performed and discussed in the experimental part of this thesis (paper V) investigated the effects of a mixture of isoflavones, the following sections focus primarily on this class of phytoestrogens.

Phytoestrogen exposure

Soybeans and soy foods are the major dietary sources of isoflavones. In soybeans isoflavone levels as high as 3810 mg/kg have been detected, whereas products such as tofu yogurt and tempeh burgers usually contain 6-20% of the amount found in whole soybeans (Kurzer & Xu 1997, Fletcher 2003). Not only soy products contain considerable amounts of isoflavones. High levels (up to 5% of the dry weight) of genistein, daidzein, biochanin A and formononetin have been found in clover (Adams 1995, Beck *et al.* 2005). Hence, clover may be an important source in grazing animals and in humans using red clover dietary supplements. Based on the content in different foods, the daily isoflavone intake has been estimated to 3.3 mg/day for average consumers and approximately 10-11 mg/day for high consumers (Clarke & Lloyd 2004).

Lignans are more ubiquitous than isoflavones and are found in varying concentrations in numerous plant foods. Flaxseed is particularly rich in lignans, but high levels are found also in whole-grain cereals (wheat, oats, rye) and certain vegetables such as asparagus, squash and carrot (Thompson *et al.* 1991, Wang 2002, Lampe 2003). Coumestans, on the other hand, are found predominantly in alfalfa and clover sprouts (Steinshamn *et al.* 2008).

Flavonoids
- Flavanones
  - 4´,7-Dihydroxyflavanone
  - Naringenin
- Flavones
  - Apigenin
  - 4´,5-Dihydroxyflavone
  - 4´,6-Dihydroxyflavone
- Flavonols
  - Kaempferol
- Hydroxychalcones
  - Phloretin
  - Isoliquirtigenin
  - 4,4´-Dihydroxychalcone
- Isoflavonoids
  - Isoflavones
    - Daidzein
    - Formononetin
    - Genistein
    - Biochanin A
  - Isoflavanones
    - O-Desmethylangolensin (O-DMA)
  - Isoflavans
    - Equol
  - Coumestans
    - Coumestrol

Lignans
- Enterolactone
- Enterodiol

Phytosterols
- β-sitosterol

Terpenoids
- Tschimganidine

Mycoestrogens*
- Zearalenone
- Zearalenol
- Zearalanol (zeranol)

Figure 2. Hierarchy and classification of phytoestrogens (Modified from Patisaul & Whitten 2005, Vajda & Norris 2006). *Metabolites of fungal species and not intrinsic plant compounds.

Concentrations of isoflavones have been determined in many human biological matrices, including urine, serum, breast milk and feces. At present, urinary and serum/plasma isoflavones are considered to be the most appropriate biomarkers of dietary exposure to this class of phytoestrogens (Lampe 2003). The isoflavone levels, in urine and blood, differ markedly between different populations. The single most important reason for this is different dietary habits. Morton and colleagues compared serum concentrations of isoflavones and in adult Japanese men and women with a similar British group (Morton *et al.* 2002). As expected considering their soy-rich diet, Japanese men and women had markedly higher serum isoflavone concentrations than subjects from the UK. The mean concentrations of genistein and daidzein in Japanese men were 492.7 nM and 282.5 nM, respectively. The corresponding concentrations in British men were 33.2 nM and 17.9 nM. In contrast, no significant difference in serum lignan (enterolactone) concentrations was found between the populations. These results are consistent with several other studies reporting very high urinary and plasma isoflavone concentrations in the Japanese population, as compared to American and European citizens (Adlercreutz *et al.* 1991, Adlercreutz *et al.* 1994, Uehar *et al.* 2000, Lampe 2003). High levels of lignans (e.g enterolactone and enterodiol), on the other hand, have been detected in vegetarians (Adlercreutz *et al.* 1994).
In addition, analysis of demographic data has revealed significant differences between subgroups within the same population. Valentin-Blasini and co-workers measured phytoestrogen concentrations in ~ 2500 urine samples from the U.S population and discovered that adolescents had significantly higher concentrations of genistein and equol than adults. They also noted that urine samples from Mexican Americans contained less equol, O-desmethylangolensin (O-DMA), enterolactone and enterodiol than Caucasian samples (Valentin-Blasini *et al.* 2005). Importantly, although most of the differences can be attributed to dietary habits it is likely that variations in phytoestrogen metabolism contribute. One study used a 3-day soy challenge to examine the capacity among Korean American and Caucasian American women to produce O-DMA and equol. The prevalence of "O-DMA producers" was significantly lower (84 vs 92%) and the prevalence of "equol producers" significantly higher (51 vs 36%) in Korean American than in Caucasian American women (Song *et al.* 2006).

Phytoestrogen metabolism

Isoflavones are found mainly in soy and red clover, where they occur primarily as glycosides (e.g genistin and daidzin). Apart from glycosides, isoflavones are found in three different forms: aglycones (unconjugated), acetylglycosides and

malonylglycosides (Cavaliere *et al.* 2007). Once ingested, genistin and daidzin get rapidly metabolized in the gut by β-glucosidases to their corresponding bioactive aglycone form, i.e genistein and daidzein, a process believed to greatly facilitate the intestinal absorption of these compounds (Setchell *et al.* 2002, Tomar & Shiao 2008). Genistein and daidzein can also be formed by bacterial demethylation of biochanin A and formononetin, respectively (Hur & Rafii 2000). Genistein is further metabolized to dihydrogenistein, which is subsequently converted into 6´-hydroxy-O-DMA (6´-OH-O-DMA) (Kurzer & Xu 1997, Heinonen *et al.* 1999). Daidzein, on the other hand, is metabolized by intestinal bacteria to the more potent metabolites equol and O-DMA (Atkinson *et al.* 2005). Importantly, there is a considerable interindividual variation in the metabolism of daidzein. Studies have revealed that 80-90% of humans are able to convert daidzein into O-DMA, whereas only 30-50% of the population can produce equol from daidzein (Lampe *et al.* 1998, Frankenfeld *et al.* 2005). Given the different properties of equol and daidzein, with regard to estrogenic potency, such a variation may be of significant toxicological importance (Schmitt *et al.* 2001).

Lignans are considered to undergo a similar metabolic processing, with bacterial and food-derived β-glucosidases converting lignan glycosides into the more active aglycones (Lampe 2003). Following intestinal absorption, lignans as well isoflavones (and their metabolites) undergo hepatic glucuronidation and sulfation. The glucuronide and sulfate conjugates are excreted in the urine and bile or undergo enterohepatic circulation (Wang 2002, Tomar & Shiao 2008).

Reproductive effects of phytoestrogens: insights from animal models

Phytoestrogens have varying binding affinities for estrogen receptors (ERs) The mycoestrogens zearalenone and zearalenol and the flavonoids coumestrol and 4´,7-diydroxyflavanone show relatively high affinity for ERs, whereas daidzein, genistein, equol and O-DMA are less potent (Shutt & Cox 1972, Miksicek 1993, Collins *et al.* 1997, Leffers *et al.* 2001). However, in comparison with 17β-estradiol and DES, phytoestrogens exhibit weak estrogenic properties. Leffers and colleagues assessed estrogenic potency of phytoestrogens by measuring the expression levels of endogenous estrogen-regulated genes in human MCF7 cells, and found that genistein was 4-6 orders of magnitude less potent than 17β-estradiol (Leffers *et al.* 2001). Collins and co-workers found that coumestrol and genistein induced an estrogenic response (i.e ER-dependent gene transcription) equivalent to 17β-estradiol only at 100 and 1000 times higher concentrations (Collins *et al.* 1997). DES is

approximately 10-100 times more potent than coumestrol, genistein and equol (Mueller *et al.* 2004).

The capacity of ERs to bind phytoestrogens, and several other classes of chemical substances, is partly attributed to the large size of the ligand-binding cavity, which is twice the volume of 17β-estradiol (Mori *et al.* 2000). Of great importance is also the structural similarity between phytoestrogens and endogenous estrogens. Isoflavonoids are particularly similar to 17β-estradiol, whereas lignans display less similarity. In contrast to 17β-estradiol, which has the same binding affinity for both ERα and ERβ, most phytoestrogens bind preferentially ERβ (Bovee *et al.* 2004, Zhu *et al.* 2006a). For example, Zhu and colleagues discovered that genistein had almost as high affinity for human ERβ as 17β-estradiol, but drastically lower affinity for ERα (Zhu *et al.* 2006a). This observation may be of significant importance since the tissue distribution differs markedly between ERα and ERβ. The two subtypes are believed to exert tissue-selective actions; ERα being more important in e.g the mammary gland and ERβ being more important in the central nervous system, the cardiovascular system and urogenital tract (Bovee *et al.* 2004).

Several cell types in the gonads of both sexes express estrogen receptors, with ERβ possibly being the more important. Although granulosa and theca cells of several species (e.g hamster, human) are positive for both ERα and ERβ (Jakimiuk *et al.* 2002, Yang *et al.* 2002), knockout experiments have shown that the β subtype is the most crucial. Couse and colleagues discovered that ovarian function (i.e follicle rapture) was severely affected in ERβ-null mice, but essentially unaffected in ERα-null mice (Couse *et al.* 2005). The testicular expression of estrogen receptors differs between the fetal and adult testis. In the testis of adult humans, ERβ has been detected in Leydig, Sertoli and germ cells, whereas ERα expression is restricted to Leydig cells (Pelletier & El-Alfy 2000, Saunders *et al.* 2001). The fetal testis also shows expression of ERβ in Leydig, Sertoli and germ cells, but ERα is absent in all testicular cell types (Gaskell *et al.* 2003, Boukari *et al.* 2007).

Apart from the previously mentioned infertility in Australian sheep, phytoestrogens have been discovered to reduce the fertility in female captive cheetahs fed a soy-based diet, which contained high levels of genistein and daidzein (Setchell *et al.* 1987). Genistein has also been suggested to influence the breeding success of California quail (Leopold *et al.* 1976). These findings demonstrate that environmental concentrations of phytoestrogens are high enough to detrimentally affect reproductive functions. Since then, animal models have demonstrated that several other phytoestrogens, including coumestrol and zearalenone, also cause adverse developmental and/or reproductive effects in both sexes. Like phthalates, phytoestrogens can cause effects during fetal/neonatal life as well as adulthood.

Perinatal exposure to phytoestrogens may alter reproductive tract formation, disturb sexual behaviour or affect pituitary function, depending on substance, timing of exposure and species studied. Wisniewski and co-workers reported that male offspring of rats fed a diet containing 5 mg/kg genistein, during gestation and lactation, had shortened AGD, delayed preputial separation and lowered testosterone concentrations in adulthood (Wisniewski *et al.* 2003). In addition, perinatal genistein exposure disturbs reproductive behaviour, making exposed males less likely to mount and ejaculate in mating tests, and affects spermatogenesis (Delclos *et al.* 2001, Wisniewski *et al.* 2003). Decreased AGD and delayed pubertal onset are also seen in the female offspring of genistein-treated rats (Levy *et al.* 1995). In contrast, genistein exposure (100 mg/kg b.w) restricted to the lactational period (PND 1-21) accelerates vaginal opening, increases uterine weight and causes a prolonged estrus in female rats (Kouki *et al.* 2003). In addition, when administered neonatally (PND 1-10) genistein has a dose-dependent biphasic effect on pituitary responsiveness (i.e GnRH-induced LH secretion) in both sexes (Faber & Hughes 1991).

Daidzein appears to be less potent than genistein during fetal and neonatal life, but possibly more potent during adulthood. Lamartiniere and colleagues reported that AGD and uterine morphology was unaffected in the female offspring of daidzein-exposed (250-1000 mg/kg) rats (Lamartiniere *et al.* 2002). Consistent with this, AGDs were found to be unchanged in rats administered daidzein during the neonatal period (Kouki *et al.* 2003). Daidzein may alter estrous cyclicity, but not as efficiently as genistein. Kouki and co-workers noted that two out of ten daidzein-treated rats exhibited a prolonged estrus, whereas all rats exposed to genistein showed either prolonged or persistent estrus (Kouki *et al.* 2003). However, in sexually mature animals the estrogenic potency of daidzein, relative to genistein, seems to be markedly higher. Female mice receiving isflavone concentrates with a ratio of 10:1 genistein:daidzein (G:D) were essentially unaffected, whereas dramatic estrogenic effects were detected in both males and females given the 1G:10D concentrate (Cline *et al.* 2004). In addition, Pan and co-workers reported that daidzein exposure (20 mg/kg b.w) throughout the puberty impaired erectile function and lowered plasma testosterone concentrations in adulthood (Pan *et al.* 2008). Pubertal exposure to genistein, on the other hand, has been found to cause relatively mild alterations, such as Leydig cell hyperplasia and a slight (non-significant) decrease in sperm count (Lee *et al.* 2004b). Genistein exposure in adulthood does not induce lesions in the testis, epididymis or prostate and lacks effect on serum LH and testosterone concentrations (Lee *et al.* 2004a, Svechnikov *et al.* 2005). However, as shown by Svechnikov and colleagues, Leydig cells isolated from adult genistein-treated rats exhibit a reduced steroidogenic response to hCG, as a consequence of down-regulated

p450scc expression (Svechnikov *et al.* 2005). Consistent with this, Opalka and co-workers found that genistein (5-50 µM) had an inhibitory effect hCG-induced testosterone synthesis in primary rooster Leydig cells (Opalka *et al.* 2004).
Interestingly, when tested in mice, genistein caused an induction of ER-dependent transcription and influenced the expression of ERα and ERβ in several tissues, such as testis, liver and lung (Montani *et al.* 2008). The same study reported that estrogen response element (ERE)-mediated transcriptional activity was induced by genistein in adults as well as in suckling pups of treated mothers. *Ex vivo* exposure of fetal testis (E14.5) to 1 µM genistein also induced ERE-mediated transcriptional activity, demonstrating that this phytoestrogen has estrogenic activity throughout the mouse development.

Possible reproductive effects in humans

A limited number of studies have been conducted in order to examine the effects of phytoestrogens on reproductive health in men, women and infants. These investigations indicate that phytoestrogen exposure during adulthood has no effects on pituitary hormone concentrations and semen parameters (concentration and motility), but possibly lowers serum testosterone concentrations and causes a very modest increment of menstrual cycle length (Mitchell *et al.* 2001, Kurzer 2002, Gardner-Thorpe *et al.* 2003). Due to the well-known detrimental effects on reproductive development seen after perinatal phytoestrogen exposure (see above), concern has been raised regarding the possible impact of these compounds on the development in humans. Strom and colleagues performed a study where self-reported pubertal maturation and reproductive history were related to the type of food (soy formula or cow milk formula) during infancy (Strom *et al.* 2001). They found no significant differences in any reproductive end-point between the diet groups, but noted a slight tendency towards longer duration of menstrual bleeding in women fed the soy-based diet. In contrast, Wolff reported a significant inverse correlation between urinary isoflavone concentrations (daidzein and genistein) and breast development in 9-year-old American girls (Wolff *et al.* 2008). However, since urinary isoflavone content is known to reflect very recent rather than long-term exposure, definite conclusion are hard to draw. Also, the sample size included in the study was relatively small.
Clearly, further studies are needed to verify the effects of phytoestrogen exposure on pubertal development as well as reproductive parameters in the adult.

Regulation of testicular steroidogenesis

Testicular steroidogenesis is essential since androgens are necessary for male sexual differentiation, pubertal development and spermatogenesis. Testosterone and dihydrotestosterone (DHT) are the major functional androgens. Whereas DHT is synthesized in Sertoli cells and outside the testis, testosterone is almost exclusively synthesized in the Leydig cells. Testosterone production is dependent on stimulation by LH secreted from the pituitary gland in response to hypothalamic GnRH. The LH receptor is a G-protein coupled receptor and the binding of LH leads to activation of adenylate cyclase, and thereby an increased cAMP production (Figure 3). cAMP has two principal functions in the control of Leydig cell testosterone synthesis. The first one is acute and involves the transfer of cholesterol from the outer to the inner mitochondrial membrane. This step is considered rate-limiting in steroidogenesis and is accomplished by the action of StAR and possibly the peripheral-type benzodiazepine receptor (PBR). The significance of StAR in steroidogenesis was confirmed when it was found that mutations in the StAR gene caused congenital lipoid adrenal hyperplasia (Bose *et al.* 1998). However, an interaction between StAR and PBR appears to be necessary for fully functional steroidogenesis, and Leydig cells with a disrupted PBR gene produce only a minimal amount of steroids even in the presence of StAR (Papadopoulos *et al.* 1997). The second function of cAMP is more prolonged and involves chronic stimulation of the enzymes required for testosterone biosynthesis from cholesterol. The first enzymatic step in testosterone synthesis is the conversion of cholesterol to pregnenolone, catalyzed by p450scc. Once formed, pregnenolone diffuses out of the mitochondrion and is translocated to the smooth endoplasmic reticulum (SER). In the SER pregnenolone is converted to progesterone by the action of 3β-hydroxysteroid dehydrogenase (3β-HSD). The next two conversions are catalyzed by the same enzyme, p450c17, possessing either 17α-hydroxylase or 17,20-lyase activity. First progesterone is hydroxylated to 17α-OH-progesterone, which is followed by the formation of androstenedione. Finally androstenedione is converted to testosterone by the action of 17β-hydroxysteroid dehydrogenase (17β-HSD). This pathway is termed Δ^4 and is the predominant one in rodents (Mathieu *et al.* 2002). P450c17 may also mediate testosterone production by converting pregnenolone to dehydroepiandrosterone (DHEA) (the Δ^5 pathway), which appears to be the major pathway in humans (Fluck *et al.* 2003).

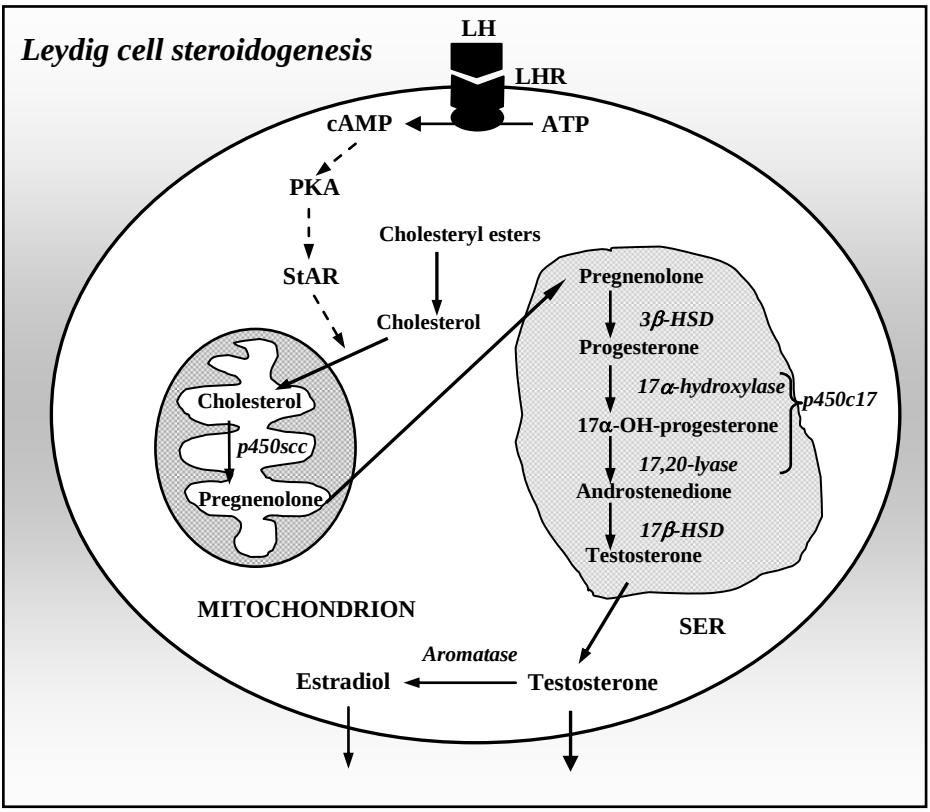

Figure 3. Schematic illustration of LH-stimulated steroidogenesis in Leydig cells. The binding of LH to its receptor (LHR) activates adenylate cyclase. This in turn increases the cAMP level, which leads to an activation of StAR. StAR then mediates the transfer of cholesterol to the inner mitochondrial membrane, where cholesterol is converted to pregnenolone in a reaction catalyzed by p450scc. Recent studies also indicate a role for PBR in cholesterol transport (not shown in the figure). Once formed, pregnenolone is translocated to SER, where it is converted in a series of reactions to testosterone. Testosterone either diffuses out of the Leydig cell or is aromatized to estradiol. Only the Δ^4 steroidogenic pathway is shown in the figure since it is the predominant pathway in rodents. Androgen synthesis may also occur via the Δ^5 pathway, as described in the text (Modified from Zirkin & Chen 2000, Haider 2004).

In the Δ^5 pathway, 17β-HSD catalyzes the conversion of DHEA to androstenediol. Androstenediol is then acted on by 3β-HSD to produce testosterone. Importantly, 3β-HSD can convert also the other Δ^5 steroids to their corresponding Δ^4 steroids. In addition to the androgen producing capacity, Leydig cells have the capacity to synthesize estrogens. This can be accomplished since Leydig cells express the enzymatic complex aromatase, which consists of two proteins: 1) cytochrome P450 aromatase (P450arom) and 2) NADPH P450 reductase (Damber *et al.* 1983, Carreau *et al.* 1999). The aromatase complex is expressed in several tissues and converts androgens to estrogens. The conversion is irreversible and thought to be under LH control, since the promoter region of the P450arom gene contains cAMP-responsive elements (CREs) (Zhou & Chen 1999). The precise role of testicular estrogens is not yet understood, but they have been suggested to influence specific stages in germ cell development (Carreau *et al.* 1999).

Steroidogenesis is apart from stimulatory signals also subject to negative regulation. The most important antisteroidogenic compound in the corpus luteum is $PGF_{2\alpha}$. The negative effects of $PGF_{2\alpha}$ on steroidogenesis are mediated by the protein kinase C (PKC) second messenger pathway and comprise diminished LH receptor synthesis, reduced cAMP accumulation, suppression of StAR and decreased mRNA expression of P450scc and 3β-HSD (Khan *et al.* 1979, Bjurulf & Selstam 1996, Fiedler *et al.* 1999, Juengel *et al.* 2000, Niswender *et al.* 2000). Of these mechanisms, the inhibitory influence on StAR is considered the primary one (Niswender *et al.* 2000). $PGF_{2\alpha}$ reduces steroidogenesis also in the testis, but the mechanisms have not been clarified to the same extent as in the corpus luteum (Saksena *et al.* 1973, Didolkar *et al.* 1981). However, Frungieri and co-workers recently reported that $PGF_{2\alpha}$ inhibited *in vitro* hCG-induced testosterone synthesis by down-regulating StAR and 17β-HSD (Frungieri *et al.* 2006).

A number of thorough reviews of Leydig cell steroidogenesis and its regulation have been published (Payne & Youngblood 1995, Habert *et al.* 2001, Haider 2004).

AIMS OF THIS THESIS

Reduced fertility and reproductive tract malformations have severe consequences for the individual as well as the society. Hence, it is of crucial importance to identify and characterize reproductive toxicants. However, risk assessment of compounds affecting the endocrine system is a challenging task for a number of reasons. The outcome of exposure is highly dependent on dose, amounts of endogenous hormones and timing of exposure. Furthermore, many endocrine disruptors are likely to produce additive effects. For these reasons it is necessary to gain further insight into their mechanisms of action. This thesis aimed at identifying mechanisms underlying the effects of cadmium, phthalates and phytoestrogens on testicular steroidogenesis.

RESULTS AND DISCUSSION

This section gives a summary and discussion of the main results from the publications (I-V) included in this doctoral thesis. Details about methodology and results are found in the publications.

Effects of Cd on the initial steps in gonadotropin-dependent testosterone synthesis (Paper I)

This study was an investigation of the temporal and dose-dependent effects of Cd exposure on the initial steps in gonadotropin-dependent testicular steroidogenesis. We performed dose-response as well as temporal-respone experiments, using adult male Sprague-Dawley rats. Cd was administered subcutaneously and the rats were sacrificed by decapitation. Testicular LHR mRNA expression and cAMP levels were measured. Administration of 10 µmol/kg Cd reduced testicular LH receptor mRNA expression by approximately 40%, as compared to saline-injected controls. No effect was found at the lower doses, 1 and 5 µmol/kg. The temporal-response experiment revealed that LH receptor mRNA was decreased after 48 h. The reduction persisted at 144 h. The same Cd dose (10 µmol/kg) caused a marked reduction of cAMP, to approximately 25% of control value at 48 h. After 144 h the cAMP level in the Cd-treated group was about 10% of the control level.

The finding by Laskey and Phelps that low-dose exposure to Cd affects testosterone synthesis has led to efforts in identifying the mechanisms underlying this effect (Laskey *et al.* 1984). Cd-induced effects on testicular steroidogenesis have been investigated both *in vivo* and *in vitro*. Laskey and Phelps suggested from *in vitro* studies that the inhibitory sites of action are subsequent to the LH receptor and cAMP synthesis, but prior to P450scc (Laskey & Phelps 1991). However, more recent data indicate that Cd can affect sites prior to cAMP as well as subsequent to p450scc. Priya and colleagues observed that Cd treatment impaired the binding of LH to its receptor in granulosa cells, and Cd exposure has been found to reduce the activity of both 3β-HSD and 17β-HSD (Biswas *et al.* 2001, Priya *et al.* 2004, Sen Gupta *et al.* 2004a).

Our study provides the first evidence that Cd can inhibit LH receptor mRNA expression. The Cd-induced reduction of cAMP is the first observation from gonadal steroidogenic cells, but has previously been described in adrenal cells (Mgbonyebi *et al.* 1994). The mechanism of action demonstrated in this paper and paper II (i.e down-regulation of StAR), has later been confirmed in ovarian steroidogenic cells. Zhang and Jia demonstrated that Cd inhibits ovarian

progesterone synthesis by reducing cAMP and down-regulating StAR gene expression (Zhang & Jia 2007). In addition, they reported that co-treatment with 8-bromo-cAMP blocked the suppressive effect of Cd on progesterone secretion and (to a large degree) StAR expression, indicating that the effect on StAR is secondary to cAMP reduction. This hypothesis is supported by the observation that StAR expression is tightly regulated by cAMP (Manna *et al.* 2003).

Noteworthy is that LH receptor mRNA expression and cAMP levels were reduced simultaneously in our experiment. The more pronounced reduction of cAMP indicates one or more amplification steps in the steroidogenic pathway, or alternatively that Cd has multiple sites of action (Laskey & Phelps 1991).

Interestingly, there is growing evidence for a stimulatory effect of Cd on steroid synthesis. Zeng and colleagues reported that long-term oral Cd exposure increased serum testosterone levels in rats (Zeng *et al.* 2003). This is in agreement with mechanistic studies. In Leydig cells, as well as in granulosa cells, low-level Cd exposure can up-regulate the expression of p450scc (Sen Gupta *et al.* 2004b, Smida *et al.* 2004). In addition, Laskey and Phelps found that Cd stimulated Leydig cell steroidogenesis when it was induced with 20α-hydroxycholesterol or pregnenolone.

To conclude, Cd can act at several steps in steroidogenesis. Its inhibitory effect on gonadotropin-dependent testosterone synthesis is likely to occur as a consequence of reduced LH binding, LH receptor expression and cAMP signalling. *In vitro* experiments indicate that the sites of stimulation are subsequent to cAMP, but further studies are needed. Future investigations should also address whether or not the reduced steroidogenesis is related to apoptosis found in other testicular cell types after Cd exposure. Testosterone is a known germ cell survival factor and this possibility should be examined.

Induction of testicular $PGF_{2\alpha}$ by Cd: protective effects of Zn (Paper II)

The aim of this study was to investigate the influence of Cd on testicular $PGF_{2\alpha}$ and testosterone synthesis. In addition, we measured the StAR protein expression in order to investigate whether this rate-limiting step in steroidogenesis was affected. Dose-response and temporal-response experiments were performed, as well as a combination experiment. In the combination experiment we examined the possible protective effect of Zn on Cd toxicity. Adult male Sprague-Dawley rats were used in all three experiments. Cd was administered subcutaneously and the rats were sacrificed by decapitation. Trunk blood was collected for testosterone measurements.

Leydig cells were isolated for subsequent analysis of StAR protein expression and testicular prostaglandins were extracted from frozen tissue prior to $PGF_{2\alpha}$ measurements.

Treatment with 10 µmol/kg Cd was found to cause a pronounced, approximately 12-fold, increase of testicular $PGF_{2\alpha}$. No effect was detected at the lower Cd doses, 1 and 5 µmol/kg. Measurements of plasma testosterone revealed that testosterone synthesis was almost completely inhibited after exposure to 10 µmol/kg Cd. Although a gradual, dose-dependent, testosterone reduction was found also in rats treated with lower doses of Cd these differences were not statistically significant. From the temporal-response experiment it became evident that the induction of $PGF_{2\alpha}$ occurred after 48 h and persisted (although less pronounced) after 144 h. The $PGF_{2\alpha}$ induction coincided with the inhibitory effect on testosterone that was noticed after 48 h and 144 h. In the combination experiment administration of 20 µmol/kg Cd gave rise to the same effects as 10 µmol/kg Cd in the dose-response experiment, i.e. a pronounced induction of testicular $PGF_{2\alpha}$ and a sharp decline in testosterone synthesis. Pre-treatment with 1 mmol/kg Zn was found to fully protect against the effects induced by 20 µmol/kg Cd. $PGF_{2\alpha}$ levels and testosterone concentrations in this group were statistically no different from saline-injected controls. Additionally, we discovered that Cd exposure (10 µmol/kg) resulted in a down-regulation of StAR protein expression.

The negative influence of $PGF_{2\alpha}$ on luteal progesterone synthesis is well established in several species and is believed to occur as a consequence of PKC activation (Niswender & Nett 1994). The antisteroidogenic effect of $PGF_{2\alpha}$ is brought about by reducing LH receptor and StAR expression and decreasing the level of cAMP (Khan *et al.* 1979, Bjurulf & Selstam 1996, Fiedler *et al.* 1999). Of these mechanisms the down-regulation of StAR protein expression has been suggested to be the primary one (Niswender *et al.* 2000). Multiple potential phosphorylation sites have been identified in StAR (ovine), providing a possible mechanism for direct regulation of this protein by PKC (Niswender *et al.* 2000).

When our study was published, the effects of $PGF_{2\alpha}$ on steroidogenesis in the testis were not as well characterized as in the corpus luteum. However, repeated $PGF_{2\alpha}$ administration had been reported to reduce testosterone synthesis in rats (Saksena *et al.* 1973, Didolkar *et al.* 1981). In 2006, Frungieri and collaborators published a study clarifying the regulatory role of $PGF_{2\alpha}$ in testosterone synthesis (Frungieri *et al.* 2006). They detected $PGF_{2\alpha}$ (FP) receptors in Leydig cells of hamsters and testicular biopsies from patients with hypospermatogenesis and Sertoli cell only syndrome. Biopsies from patients with normal spermatogenesis, on the other hand, lacked FP receptor

expression. In addition, $PGF_{2\alpha}$ was found to inhibit *in vitro* hCG-induced testosterone production, by down-regulation of StAR and 17β-HSD expression.

Our study shows for the first time that Cd can induce testicular $PGF_{2\alpha}$, which might help to explain the well-known antisteroidogenic effect of this metal. This observation indicates that Cd can block testicular testosterone synthesis not only by affecting the stimulatory cAMP second messenger pathway (paper I), but also by inducing the inhibitory PKC pathway. This is consistent with earlier reports of Cd-induced PKC activation in other cell types (Block *et al.* 1992, Bagchi *et al.* 1997, Long 1997). In the corpus luteum $PGF_{2\alpha}$ activates its own synthesis (Diaz *et al.* 2002).Whether or not it occurs in the testis is not established, but Leydig cells express FP receptors and have the capacity for $PGF_{2\alpha}$ synthesis (Mather *et al.* 1983, Orlicky & Williams-Skipp 1992, Suzuki-Yamamoto *et al.* 2007). Such a positive feedback loop could explain the very high levels of $PGF_{2\alpha}$ and in turn markedly reduced testosterone production detected in our study.

Paper II was also the first to demonstrate a suppressive effect of Cd on StAR protein expression. The same year, Sen Gupta and colleagues reported that Cd exposure reduced testicular StAR mRNA levels and a recent publication described the same effect in ovaries of Cd-treated rats (Sen Gupta *et al.* 2004a, Zhang & Jia 2007). Since StAR expression is regulated by cAMP as well as $PGF_{2\alpha}$ (PKC), it is not possible to tell from our results whether the suppressive effect on StAR is caused by PKC induction or decreased cAMP concentrations. Zhang and Jia noted that addition of 8-bromo-cAMP restored, to some extent, StAR mRNA levels in Cd-treated granulosa cells, suggesting that Cd down-regulates StAR partly by reducing cAMP and partly by other mechanisms (PKC activation).

Possibly there is also an involvement of tumor necrosis factor-α (TNFα), a cytokine induced by, and in itself an inducer of, $PGF_{2\alpha}$. TNFα affects several steps in Leydig cell steroidogenesis, including cAMP production and StAR expression (Mauduit *et al.* 1991, Mauduit *et al.* 1998).

It well known that Zn can protect testicular tissue against Cd-induced damage (Parizek & Zahor 1956, Webb 1972). Our finding that Zn can protect against Cd-induced effects on $PGF_{2\alpha}$ production and testosterone synthesis is new, but may be related to earlier findings. A possible mechanism for Zn to counteract the effects of Cd is to protect against oxidative stress. Cd has been demonstrated to stimulate PGE_2 synthesis by a mechanism involving increased lipid peroxidation and arachidonic acid release (Ramirez & Gimenez 2003). Hence, it is plausible that Zn blocks $PGF_{2\alpha}$ induction by protecting against oxidative stress.

Apart from inducing Cd-binding MTs, Zn can protect sulfhydryl groups against oxidation (Bray & Bettger 1990, Powell 2000). In the liver Zn is known to reduce the lipid peroxidation caused by Cd exposure (Khan *et al.* 1991). Zousa and co-workers measured the expression of heat shock protein 70 (Hsp70) as a marker of oxidative stress in Cd-treated hepatic stellate cells, and found that Zn pre-treatment prevented the stimulatory effect of Cd on Hsp70 (Souza *et al.* 2004). However, the protective capacity of Zn against lipid peroxidation seems to vary between different tissues (Khan *et al.* 1991).
Cd toxicokinetics are also known to be altered by Zn and Waalkes and Perantoni concluded from *in vitro* experiments that addition of Zn markedly reduces the cellular uptake of Cd in isolated Leydig cells (Waalkes & Perantoni 1988). In addition, Zn status has been found to influence the number of binding sites for $PGF_{2\alpha}$ in membranes, which may be related to the effects found in our investigations (Li & O'Dell 1986).

Cd induces GAPDH gene expression but does not influence the expression of adrenergic receptors in the testis (Paper III)

In this study, we evaluated the effect of Cd exposure on the expression of adrenergic receptors and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the rat testis. Sprague-Dawley rats received subcutaneous injection of 10 µmol/kg Cd and were sacrificed by decapitation 0.48-144 h later. Testicular mRNA expression of α_{1A}- and β_2-adrenergic receptors (α_{1A}-AR and β_2-AR) and GAPDH was analyzed. A statistically significant (~ 1.6 fold) up-regulation of GAPDH mRNA expression was detected 48 h after exposure to Cd. The elevated mRNA level persisted 144 h post-exposure. In contrast, the expression of α_{1A}- and β_2-ARs did not differ between Cd-treated rats and control animals.
Our observation that Cd can induce GAPDH mRNA expression is new, but other transition metals (i.e cobalt, nickel and manganese) have previously been demonstrated to stimulate GAPDH expression (Graven *et al.* 1998, Hazell *et al.* 1999).
Historically, GAPDH was considered to be of limited importance beyond basic cell catabolic processes (Sirover 1997). At present, GAPDH is suggested to have a role several biological processes, including mRNA regulation, DNA repair and initiation of apoptosis (Sirover 1999, Berry & Boulton 2000). Interestingly, GAPDH expression appears to be regulated by androgens. Ripple and Wilding noted that androgen treatment reduced GAPDH mRNA expression in prostate cancer LNCaP cells, whereas androgen withdrawal has been reported to increase GAPDH levels (Ripple & Wilding 1995, Epner *et al.*

1999) . These findings made us speculate (in paper III) that GAPDH induction occurred as a consequence of reduced testosterone concentrations in our experiments. Since our paper was published, GAPDH has been shown to be an important regulator of androgen receptor function (Harada *et al.* 2007). Harada and colleagues recently identified GAPDH as a potent coactivator of androgen receptor-mediated transcription. They also found that ERα transcriptional activity was unaffected by GAPDH; demonstrating a high specificity for the androgen receptor. Hence, androgen deprivation appears to result in the induction of GAPDH, which in turn elicits a compensatory response by increasing androgen receptor transcriptional activity. Such a mechanism may explain why anti-androgens sometimes fail to suppress prostate tumor growth (Harada *et al.* 2007). Indeed, significantly elevated GAPDH mRNA levels have been detected in prostatic neoplasms (Sharief *et al.* 1994). Cd exposure causes prostatic neoplastic lesions in laboratory animals and the possible involvement of GAPDH ought to be analyzed (Waalkes *et al.* 1992).

Testicular functions are also dependent on GAPDH and male mice lacking GAPDH expression are infertile with severe defects in sperm motility (Miki *et al.* 2004). In addition, nuclear translocation of GAPDH has been proposed as an early and important mechanism in the initiation of apoptosis (Kusner *et al.* 2004). Hence, future studies should be directed at further characterizing the role of GAPDH in physiological and pathophysiologal conditions as well as its role in Cd toxicity.

Our study also revealed that Cd-induced suppression of testosterone synthesis (described in paper II) does not involve an altered gene expression of α_{1A}- and β_2-ARs. This finding suggests that the influence of Cd on testicular gene expression involves a specific effect on the LH receptor and not a general effect on seven-transmembrane-spanning receptors. Testicular steroidogenesis, which is controlled mainly by gonadotropins, can be influenced also by catecholamines. Catecholamines stimulate steroid biosynthesis in isolated Leydig cells as well as whole decapsulated testes, but appear to have the opposite function in Cd toxicity (Anakwe & Moger 1984, Anakwe *et al.* 1985). Biswas and colleagues described a close correlation between increased catecholamine levels and decreased testosterone synthesis after Cd exposure (Biswas *et al.* 2001). Our data indicate that the inhibitory activity of catecholamines is not due to altered expression of adrenergic receptors but is explained by other mechanisms. Such mechanisms may be a changed testicular blood flow or a direct effect on the Leydig cells (e.g via adrenergic receptor signalling).

Stimulatory effect of MEHP on basal gonadal steroidogenesis *in vitro* (Paper IV)

In this study we investigated the effects of MEHP on gonadal steroidogenesis *in vitro*. High-dose exposure to phthalates is known a to suppress testosterone synthesis in laboratory animals. In addition, an inhibitory effect on hCG-induced steroid synthesis has been described *in vitro*. Our intention was to examine the impact of phthalate exposure on basal gonadal steroidogenesis. For this purpose mouse Leydig tumor cells (MLTC-1) and KK-1 granulosa tumor cells were used. We discovered that 25-100 µM MEHP stimulated basal steroidogenesis in a concentration-dependent manner in MLTC-1. Gene and protein expression analsyses of key steroidogenic proteins revealed that MEHP exerts its stimulatory effect by a cAMP- and StAR-independent mechanism. MEHP, in the concentration range 10 to 100 µM, up-regulated hormone-sensitive lipase (HSL) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene expression, suggesting that MEHP increases the amount of cholesterol available for steroid synthesis. Also in KK-1, MEHP was found to stimulate basal steroidogenesis by a cAMP-independent mechanism. In addition, we confirmed the inhibitory action of MEHP on hCG-induced steroid synthesis.

The present study provides novel evidence for a direct stimulatory effect of MEHP on gonadal steroidogenesis *in vitro*. During the final preparation of our article, Ge and colleagues published a study describing a similar effect in primary rat Leydig cells, indicating that the mechanism of action reported by us reflects the situation in primary steroidogenic cells (Ge *et al.* 2007). Our article is the first to report an induction of steroid synthesis by MEHP in female gonadal cells. The findings presented in this paper suggest that MEHP, besides its known inhibitory effect on hCG action, can stimulate basal steroidogenesis in both sexes. Interestingly, the stimulatory effect was demonstrated at MEHP concentrations that have been recorded in the plasma of exposed newborn children (Sjoberg *et al.* 1985). Our results contribute to the understanding of the developmental and reproductive toxicology of phthalates.

Most animal studies investigating *in utero* exposure to DEHP and DBP have used doses exceeding 100 mg/kg/day. Such high-dose exposure causes a sharp decrease in testicular testosterone synthesis and gives rise to hypospadias, cryptorchidism and malformations of the prostate and testis (Mahood *et al.* 2005, Foster 2006). Consistent with this, Swan and co-workers discovered reduced anogenital distance and impaired testicular descent in boys of mothers with elevated phthalate exposure during pregnancy (Swan *et al.* 2005).

At doses relevant to human exposure, DEHP appears to cause more subtle alterations, such as increased testosterone secretion (Andrade *et al.* 2006b). Increased testosterone concentrations have been detected after exposure to doses as low as 0.045 mg/kg/day (Andrade *et al.* 2006b) Hence, the effects described in our paper reflect low-dose rather than high-dose exposure. Phthalate-induced increment of steroid hormone levels has been proposed to influence pubertal development. Ge and colleagues found that prepubertal exposure to 10 mg/kg/day DEHP was associated with increased serum testosterone concentrations and advanced pubertal onset in rats (Ge *et al.* 2007). A similar effect on pubertal development and hormone concentrations has been demonstrated also in female rats (Ma *et al.* 2006). In addition, a Puerto Rican study revealed that 68% of girls with premature breast development had elevated serum concentrations of DEHP, MEHP and DBP (Colon *et al.* 2000). Our observation that MEHP has a direct stimulatory effect on steroidogenesis in Leydig as well as granulosa cells supports and strengthens the hypothesis that phthalates may accelerate pubertal development. Whether or not phthalates alter pubertal onset in humans remains to be established. Besides the opposite effects on basal and hCG-induced steroid production (paper IV), DEHP produces different effects depending on dose. In contrast to low dose treatment (see above), 750 mg/kg/day DEHP delayed preputial separation (Ge *et al.* 2007). Hence, a correct assessment of the impact of phthalates on human steroidogenesis and reproductive function will require in-depth knowledge of their mechanisms of action. We show in this study that MEHP induces steroid biosynthesis by a mechanism different from the one of hCG stimulation. MEHP did not influence cAMP and reduced the mRNA expression of StAR, p450scc and p450c17. In contrast, MEHP up-regulated HSL and HMG-CoA reductase gene expression. These results made us hypothesize that the effect was mediated through peroxisome proliferator-activated receptors (PPARs), which have been shown to be activated by MEHP (Lapinskas *et al.* 2005, Venkata *et al.* 2006). PPARs belong to the nuclear hormone receptor family and regulate genes in lipid metabolism (Le Jossic-Corcos *et al.* 2004, Feige *et al.* 2007). Apart from this function they are known to affect cAMP signalling, by interfering with cAMP-activated transcription factors (e.g Sp1) (Han *et al.* 2005). According to the hypothesis, MEHP activates PPARs that in turn, by inducing genes involved in cholesterol metabolism, increase the substrate available for steroid synthesis. On the other hand, activation of PPARs also leads to a down-regulation of cAMP-dependent steroidogenic genes, thus explaining why the stimulatory effect of hCG is reduced. A study by Lapinskas and colleagues together with unpublished data from our laboratory supports this theory. Lapinskas and collaborators demonstrated that all tested phthalates except DEHP, i.e MEHP, MBP and

DBP, could bind to and activate PPARs with different potency (Lapinskas *et al.* 2005). Our experiments showed that MBP and DBP, but not DEHP, affected Leydig cell steroidogenesis. In addition, it is known that PPARγ ligands can stimulate granulosa cell progesterone synthesis (Froment *et al.* 2003). Future studies should be designed to test this hypothesis.

Stimulatory effect of phytoestrogens on T_3 secretion and testicular steroidogenesis during puberty (Paper V)

This investigation was undertaken in order to elucidate the effect of low-dose dietary phytoestrogen exposure on testosterone production during puberty. Male goat kids at the age of 3 months received either 3-4 mg/kg/day isflavones, in the form of Novogen red clover tablets, or a control diet for ~3 months. At the age of 5 months, phytoestrogen-fed animals had significantly higher plasma testosterone concentrations than controls (37.5 nmol/l vs 19.1 nmol/l). The rise in testosterone was preceded by an elevation of plasma total triiodothyronine (T_3) two weeks earlier. Free T_3 concentrations were also significantly higher in the phytoestrogen group, but this effect was detected slightly later in the experiment. At the end of the experiment, we observed a slight, non-significant, tendency towards reduced testicular steroidogenesis.

The present study shows that low-dose exposure to isoflavones present in red clover can influence thyroid hormone secretion as well as gonadal steroiodogenesis. Since soy isoflavones appear not to influence thyroid hormones, this effect is likely to be specific for one or more of the isoflavones found in clover (Bruce *et al.* 2003, Dillingham *et al.* 2007, Teas *et al.* 2007). Hence, consumers of red clover extracts (e.g menopausal women) may be at higher risk than those consuming a traditional soy-based diet. Our finding is particularly interesting since the dose used is only 3-5 fold higher than the one recommended for menopausal women and men suffering from prostate enlargement. For this reason, future studies should aim at further clarifying the impact of isoflavones derived from clover on thyroid hormone secretion. An increased testosterone synthesis may advance pubertal onset, as described after exposure to other endocrine disruptors, why the mechanisms of action of phytoestrogens on gonadal steroidogenesis ought to be established (Ge *et al.* 2007) .

In the present study, the mechanisms whereby isoflavones stimulate T_3 and testosterone synthesis were not established. Previous reports have demonstrated that different isoflavones possess different properties. For example, Almstrup and colleagues found that biochanin A was a potent aromatase inhibitor, whereas genistein lacked such activity (Almstrup *et al.*

2002). The tablets used in our investigation contained a large proportion of biochanin A and it is possible that a direct inhibitory effect of this isoflavone on aromatase decreases the conversion of testosterone to estradiol, thereby resulting in higher testosterone concentrations. However, since T_3 concentration was found to be increased at an earlier time-point than testosterone it seems likely that the major mechanism underlying the stimulation of steroidogenesis was an elevated T_3 secretion. Indeed, previous studies from our laboratory, as well as others, have demonstrated that phytoestrogens can increase T_3 concentrations in both animals and humans (Watanabe *et al.* 2000, Madej *et al.* 2002). T_3 has a direct stimulatory effect on Leydig cell steroidogenesis during puberty as well as adulthood in several species, including goat, mouse and rat (Antony *et al.* 1995, Jana *et al.* 1996, Manna *et al.* 1999, Maran *et al.* 2000). Consistent with this, hypothyroid rats have a reduced testosterone synthesis as compared to rats with normal thyroid function (Antony *et al.* 1995). The mechanism whereby T_3 induces steroidogenesis remains to be established, but *in vitro* studies have revealed T_3 has the ability to up-regulate StAR as well as p450scc expression (Manna *et al.* 1999, Manna *et al.* 2001). Unfortunately, we had no opportunity to analyze the expression of steroidogenic enzymes at the time-point at which the testosterone concentration was elevated in our experiment.

ERs are expressed in numerous tissues and the two subtypes (α and β) are believed to exert tissue-selective actions. ERβ is considered to be the more important subtype in the urogenital tract (Bovee *et al.* 2004). Couse and co-workers found that ovarian function (i.e follicular rapture) was severly affected in ERβ-null mice, but essentially unaffected in ERα -null mice (Couse *et al.* 2005). In the adult human testis ERβ expression has been detected in Leydig, Sertoli and germ cells, whereas the expression of ERα is restricted to Leydig cells (Pelletier & El-Alfy 2000, Saunders *et al.* 2001). Most phytoestrogens bind preferentially to ERβ, suggetsting that reproductive functions are particularly sensitive to exposure to this class of endocrine disruptors (Bovee *et al.* 2004). Consistently, genistein and daidzein have been reported to inhibit testosterone synthesis *in vivo* as well as *in vitro* (Wisniewski *et al.* 2003, Opalka *et al.* 2006, Pan *et al.* 2008). To the best of our knowledge, direct effects of biochanin A on Leydig cell steroidogenesis have not been investigated. Biochanin A has a significantly lower affinity for ERs than genistein and daidzein, but has been identified as a potent aromatase inhibitor (Miksicek 1994, Kuiper *et al.* 1998, Almstrup *et al.* 2002). Hence, one can expect the effects of biochanin A on steroid synthesis to differ from the ones previously described for other isoflavones.

Since biochanin A is estrogenic at higher concentrations it is possible that the discrepancy between the present study and previous ones is due to the doses

tested (Almstrup *et al.* 2002). Hence, to establish dose-repsonse curves for each isoflavone on testicular steroidogenesis would be of great value. Future studies should also compare, in detail, the effects on reproductive parameters after exposure to isoflavones derived from clover and soy isoflavones.

GENERAL DISCUSSION

Most studies show that male reproductive health is declining in industrialized countries throughout the world. Endocrine disruptors are known to influence reproductive functions in experimental animals as well as occupationally exposed workers. To assess the impact of endocrine disruptors on the reproductive health of the general population is an important and challenging task. In order make a correct assessment a number of considerations should be taken into account, including 1) combined effects of different compounds 2) dose-dependent biphasic effects 3) multiple sites of action 4) species and sex differences 5) genetic polymorphisms 6) exposure measurements and 7) parameters for reproductive development and function.
Although the properties of the toxicants investigated in this thesis differ, all of them are relevant to study with regard to human reproductive health. Due to its high toxicity and very long biological half-life, Cd is still of great interest despite the reduced usage during the last decades. Phthalates and phytoestrogens have a low acute toxicity and short biological half-life, but exposure is almost unavoidable since they occur ubiquitously in daily life products and foods.

Combined effects of different compounds

If exposure to environmental endocrine disruptors is responsible for the decline in male reproductive health, which has been suggested, it is likely to be a consequence of additive effects of numerous compounds. However, it is also possible that one compound may block the action of another or that two toxicants counteract the effects of each other.
Unfortunately, the number of reports devoted to additive/counteracting effects is relatively small. The results presented in paper II may serve as a good example. This study showed how one metal can block the detrimental effects of another. Pre-treatment with Zn was found to protect, very efficiently, against Cd-induced testosterone suppression and rise in testicular $PGF_{2\alpha}$. Additive effects have been described by Howdeshell and colleagues. They investigated the effects of a mixture containing varying concentrations of five phthalates and found that fetal testosterone synthesis was reduced in a dose-additive manner (Howdeshell *et al.* 2008). Even though not extensively studied, additive effects of are likely to occur since many endocrine disruptors induce similar effects. We found that two of the analyzed toxicants, i.e cadmium and MEHP, were able to suppress gonadal steroidogenesis, although under different conditions (papers II, IV). Also phytoestrogen exposure (long-term) caused a slight reduction of testosterone levels, but it was not significant

(paper V). Some substances not only cause alterations of the same type, but also have a common mechanism of action. Our studies revealed that Cd as well as phytoestrogens lowers testicular cAMP levels (papers I and V), whereas an up-regulation of GAPDH expression has been documented after exposure to Cd (paper III) and TCDD (McNulty & Toscano 1995). In addition, as shown in paper II, Cd inhibits steroidogenesis in part by down-regulating StAR, a mechanism seen also in lead-exposed Leydig cells (Huang & Liu 2004).

It is known that cadmium as well as phthalates and phytoestrogens can bind to ERs and induce ER-dependent transcription (Harris *et al.* 1997, Kurzer & Xu 1997, Stoica *et al.* 2000, Takeuchi *et al.* 2005). Due to the promiscuity of ERs it is highly probable that more substances will be identified as estrogenic in the future.

Dose-dependent biphasic effects

A correct interpretation of experimental data also requires awareness of the fact that some endocrine disruptors induce very different effects depending on dose, "endocrine status" and timing of exposure. We show, in paper IV, that treatment with 100 µM MEHP stimulates basal Leydig cell steroidogenesis but has the opposite effect on hCG-induced steroid synthesis. Similarly, Laskey and Phelps reported that Cd suppressed hCG-induced testosterone production, whereas pregnenolone-induced synthesis was increased (Laskey & Phelps 1991).

Dose-dependent effects have been reported for both phytoestrogens and phthalates. Faber and Hughes noted that genistein hade a dose-dependent biphasic effect on pituitary responsiveness, whereas Ge and colleagues found a dose-dependent biphasic effect of DEHP on pubertal onset (Faber & Hughes 1991, Ge *et al.* 2007). The timing of exposure to endocrine disruptors is critical to the outcome during fetal as well as postnatal life. Welsh and colleagues recently identified a fetal programming window (E.15.5-E19.5), in which androgen action programs the masculinization of all reproductive tract tissues in rats (Welsh *et al.* 2008). Only within this window, which precedes morphological differentiation, can anti-androgens induce hypospadias and cryptorchidism (Welsh *et al.* 2008). Interference with testosterone synthesis/signalling later in fetal life may instead influence the number of Sertoli cells (Scott *et al.* 2008). The opposite effects seen after *in utero* and lactational exposure to genistein illustrates that timing of exposure is important also in the female. The offspring of genistein-treated rats display delayed vaginal opening, whereas exposure restricted to the lactational period accelerates pubertal onset (Levy *et al.* 1995, Kouki *et al.* 2003).

Multiple sites of action

In vitro studies, in particular, have shown that some endocrine disruptors may affect more than one level of the hypothalamic-pituitary-gonadal (HPG) axis. As shown in paper IV, MEHP has a direct stimulatory effect on gonadal steroidogenic cells of both sexes. From other *in vitro* experiments it is known that MEHP also increases the release of LH from the pituitary (Svechnikova *et al.* 2007). Cd has also been reported to affect both pituitary function and gonadal steroidogenesis (Laskey & Phelps 1991, Poliandri *et al.* 2003). Hence it is important to establish if a toxicant acts at multiple sites *in vivo* and identify the primary site(s) of action. Due to the negative feedback regulation of the HPG axis it is difficult to identify the primary site(s) of action based on *in vivo* hormone measurements. However, further characterization of molecular mechanisms and subsequent studies in cell-specific knockout mouse models would greatly facilitate such efforts.

Species and sex differences

Many effects induced by endocrine disruptors are not associated with species or sex differences. As described in paper IV, MEHP stimulates steroidogenesis by a cAMP-independent mechanism in Leydig as well as granulosa cells. In addition, we show that Cd inhibits testicular steroid synthesis by reducing cAMP and down-regulating StAR (papers I and II), a mechanism of action later described for Cd-induced inhibition of ovarian steroidogenesis (Zhang & Jia 2007).

However, the metabolism and effects of certain toxicants differ between species, strains within a species and between individuals of the same or different sex. Ito and co-workers found that intestinal lipase activity and thus the hydrolysis of DEHP to MEHP was significantly higher in rodents than in marmosets (Ito *et al.* 2005). Additionally, in rats there is a considerable strain variation in the susceptibility to DEHP-induced cryptorchidism, with Wistar rats being much more vulnerable than Sprague-Dawley rats (Wilson *et al.* 2007). Significant individual variations in the metabolism of phytoestrogens (i.e daidzein) are known to exist among humans. Only 30-50% of the population is capable of producing equol from daidzein (Frankenfeld *et al.* 2005). The metabolism of daidzein is also a good example of gender differences; the capacity to metabolize daidzein to equol increases during a prolonged soy diet in women but not in men (Lu & Anderson 1998). Such variations must be taken into account when interpreting laboratory results and judging the possible effects on human reproduction.

Genetic polymorphisms

In recent years polymorphisms in AhR, AR and ERs have been proposed to influence the vulnerability to endocrine disruptors. AhR polymorphisms that influence the susceptibility to TCDD have been identified in mouse and rat (reviewed in Okey *et al.* 2005). In mouse, a polymorphism in the ligand-binding domain of AhR has been shown to greatly reduce binding affinity and the subsequent toxic response mediated by AhR (e.g CYP1A1 induction, teratogenicity, lipid peroxidation). Han/Wistar (H/W) rats have a 1000-fold higher resistance to TCDD-induced lethality than Long-Evans rats, due to a polymorphism that causes a deletion in the transactivation domain of AhR. Similarly, H/W rats have been shown to be less sensitive to TCDD-induced decrease in sperm number after developmental as well as adult exposure (Simanainen *et al.* 2004a, Simanainen *et al.* 2004b).

It has been suggested that polymorphisms in nuclear receptors influence vulnerability to endocrine disruption also in humans. Results obtained by Giwercman and collaborators indicate that the AR CAG repeat length modifies the susceptibility to detrimental effects on semen quality induced by 2,2´,4,4´,5,5´-hexachlorobiphenyl (CB-153) (Giwercman *et al.* 2007). Cryptorchidism has been found to be associated with homozygosity for a specific haplotype of ESR1 (the gene encoding ERα), but the relevance of this finding with regard to endocrine disruption remains to be established. The human AhR binds TCDD with relatively low affinity, possibly explaining why humans are resistant to the most adverse health effects of dioxin-like compounds (Okey *et al.* 2005). However, polymorphisms in the transactivation domain have been described and their impact on the sensitivity to dioxin-like chemicals should be further investigated (Harper *et al.* 2002).

Exposure measurements

As a consequence of its long biological half-life, exposure to Cd is relatively easy to measure in a reliable manner. Urinary Cd concentration is considered a good indicator of body burden, whereas the blood concentration, due to one "fast" and one "slow" component, can be used to determine recent as well as long-term exposure (Nordberg *et al.* 2007). To make accurate estimations of phthalate and phytoestrogen exposure are significantly more complicated. Concentrations in serum or urine of these compounds will, because of their rapid excretion, only reflect recent exposure. Exposure analyses are further complicated by the fact that many phthalates and phytoestrogens are converted into metabolites with different toxicological properties. For example, daidzein is metabolized in varying degree to the more potent metabolites equol and O-DMA (Atkinson *et al.* 2005). In addition, for certain phthalates (e.g DEHP), it is not yet fully clarified which metabolites that are responsible for causing

endocrine disruption. Hauser and colleagues reported that oxidation of MEHP to MEHHP and MEOHP was "protective" for human sperm DNA damage, whereas Stroheker and co-workers noted that MEHHP and MEOHP, but not MEHP, inhibited AR-dependent transcriptional activity (Stroheker *et al.* 2005, Hauser *et al.* 2007).

Many epidemiological studies with large sample sizes have been based on estimations of intake rather than actual exposure measurements. This method requires not only the participants to accurately recall previous intake/exposure, but also suffers from not taking into account the relative amount of bioactive metabolites. On the other hand, it enables estimations of long-term exposure to compounds with short biological half-lives.

In conclusion, accurate exposure measurements require in-depth mechanistic studies to determine the actions of each metabolite as well as development of better methods to analyze exposure to toxicants with short half-lives. Recent studies have revealed that secondary oxidized metabolites have significantly longer elimination half-lives than MEHP, thus being more suitable markers of long-term exposure to DEHP (Koch *et al.* 2006).

Parameters for reproductive development and function

The regulation of reproductive development and function is complex and not yet fully understood. In addition, there are considerable species differences. Hence, future in-depth studies of regulatory mechanisms, hormonal and non-hormonal, in humans as well as experimental animals are required for accurate risk assessment of endocrine disruptors.

We discovered (paper IV) that MEHP exposure could induce opposite effects depending on "endocrine status". MEHP reduced hCG-induced steroid synthesis, but caused a 6-fold stimulation of basal steroidogenesis. Thus, to elucidate which developmental processes and reproductive functions that are under control of gonadotropins will be essential for predicting effects in humans. During the last decades it has been established in both rodents and humans that postnatal, but not prenatal, sexual development is dependent on LH (Weiss *et al.* 1992, El-Gehani *et al.* 1998, Zhang *et al.* 2001, Ahtiainen *et al.* 2007). However, which is important for the interpretation of animal data, non-gonadotropic factors can substitute for LH in rodents, whereas hCG is believed to have this function in humans (Ahtiainen *et al.* 2007). Hence, in theory, a toxicant that specifically alters LH receptor function would not affect prenatal sexual development in rodent models, but still cause profound alterations in humans.

The recent discovery of a fetal programming window (see above) has provided new insights regarding hormonal regulation of reproductive tract development (Welsh *et al.* 2008). In addition to the findings mentioned previously, Welsh

and colleagues showed that androgen-driven masculinization of the female was confined to the same period in fetal life (i.e E15.5-E19.5). They also found that exogenous testosterone did not advance or enhance reproductive tract development in the male. To conclude, from E15.5 to E19.5 the male fetus is vulnerable to substances with antiandrogenic properties but resistant to androgen exposure. The female fetus, on the other hand, is sensitive to androgenic compounds during the same time period.

All the toxicants investigated in this thesis have been found to alter pubertal onset in experimental animals (Johnson *et al.* 2003, Kouki *et al.* 2003, Ge *et al.* 2007). In addition, phthalates and phytoestrogens have been suggested to influence breast development in girls (Colon *et al.* 2000, Wolff *et al.* 2008). However, before the signal(s) that activate the GnRH pulse generator are identified it will be hard to characterize in detail the mechanisms underlying the effect on pubertal development. Kisspeptins were recently suggested to have an important role in pubertal onset, by stimulating GnRH secretion, and future studies should address the influence of endocrine disruptors on kisspeptin synthesis and signalling (Castellano *et al.* 2006).

CONCLUDING REMARKS

We identified mechanisms underlying stimulatory and inhibitory effects of cadmium, phthalates and phytoestrogens on testicular steroidogenesis.

The specific findings were:

- Cd inhibits testicular steroidogenesis *in vivo* by:
  - Down-regulating LH receptor expression
  - Redcucing cAMP levels
  - Suppressing StAR protein expression
  - Inducing $PGF_{2\alpha}$
- Pre-treatment with Zn protects against Cd-induced effects on testosterone and $PGF_{2\alpha}$
- Cd up-regulates GAPDH expression in the testis, possibly secondary to reduced testosterone synthesis
- MEHP stimulates Leydig and granulosa cell steroidogenesis *in vitro* by a cAMP- and StAR-independent mechanism
- MEHP is likely to exert its stimulatory effect by increasing the amount of cholesterol available for steroid synthesis
- Low-dose dietary exposure to phytoestrogens present in clover increases T_3 secretion and testosterone synthesis during puberty in male goats

ACKNOWLEDGEMENTS

Först och främst vill jag tack mina handledare under åren; **Gunnar Selstam, Gunnar Nordberg** och **Per Leffler**. Ett stort tack för att ni gav mig möjlighet att doktorera inom ett så spännande område som reproduktionstoxikologi. Under era vingar har jag fått möjlighet att utvecklas både på labbet och i skrivarlyan.
Tack **Gunnar S** för stöd och entusiasm, otroliga fortplantningskunskaper och ett avväpnande ”a´ la bonne heure” emellanåt.
Tack **Gunnar N** för intressanta diskussioner om metalltoxikologi, all hjälp med kadmiumpeken och trevliga Kinaresor.
Tack **Pelle** för att du fick mig att höja blicken ovanför gonaderna… Tack vare dina insatser har jag lärt mig nya tekniker och lärt känna FOI:s toxgäng.

Tack till **Regina**, min parhäst i labbet. Toppen att du alltid ställer upp när jag inte begriper mig på elektroniska apparater och knepiga datorprogram. Hoppas du får din doktorstitel snart; vem ska von Hofsten annars diskutera med under middagarna på Riddarhuset?

Ett stort tack går även till er andra som varit involverade i projektet. **Christina**, för all hjälp med råttorna och lösningar till kurs-lab. **Gunvor**, för att du lärde mig lösningshyb. **Mona**, för bra labsamarbete och korrekturläsning av artiklar. **Birgitta,** för hjälp med att isolera Leydigceller och mycket annat. **Per Lundgren**, för stöd under de första åren i labbet. Ex-jobbarna **Gunilla** och **Emelie**, för ftalatinsatser. Gillar skarpt att du, Emelie, blivit reproduktionsforskningen trogen! Thanks **Kui Liu** for scientific input and showing that anything is possible with hard work and devotion.
Vill även tacka er andra som varit i labbet under åren. **Jenny, Annelie och Peter**; in situ-hybbar och oförglömliga Estlandsresor (tack där till **Andres, Raivo, Alar och Siim**).

Tack **Andrzej Madej** för gott samarbete med manuset och för att du hjälper mig när jag är är nyfiken på getter.

Ett nostalgisk tack går till **Zoofysgänget**, med **Jonas** & **Lisa** och **Jonas vH** i spetsen. Skönt att ni är tillbaka. Vi är ju de enda som kan göra en hjärt-lungdissektion…

Ett dagsaktuellt tack till **Fikagruppen**, både **Nilssons gäng** och **Flugfolket**. Ni gör livet på jobbet glatt. **Anna ”Ja ä int bitter” Larsson** och **Linn**, ert fikasällskap och tips om hur livet bör levas är varmt uppskattade. **Erik ”FlyBase I´m in love” Tegeling** för alla roliga squashmatcher och eftertänksamma svar. **Sa** för din omtänksamhet och nyvunna ironiska sida (tyder på att det blivit några år i Sverige vid det här laget…). **John,** för ditt lugna och balanserade sätt, det är ett bra komplement i gruppen. Ta väl hand om flickorna och Erik!
Linus och **Chaz** för skojiga fester och insikten att Skövde är lite, lite bättre än Skara. **Andreas** och **Sanna** för att ni gör det så trevligt att grilla på Bölesholmarna varje år.

Sara för att du får det ologiska att verka logiskt, och vice versa, och bjuder på state-of-the-art-bakverk. Tack **Therese** för att du livar upp partajen.

Tack **Lisa, Regina, Jonas vH, Mats, Sara, Viktoria, Jenny JS** och **Jannek** för gott samarbete och trevligt sällskap på kurslab.

Tack också till resten av **Molekylärbiologen**.

Självfallet vill jag tacka det härliga gänget med MolBiol-kursare som hängt ihop i evigheter.
Er vänskap är ovärderlig.
Peter, min vapendragare som börjar ett smörgåsbord med två gräddbakelser under förevändningen att ”lite sött retar aptiten” och anser att tid är ett relativt ting. Med dig är det alltid lika trevligt. **Johan Bossman** som tror att jag springer milen på 29.25 och till vars pipa det varit ett sant nöje att dansa. Saknar dock din himmelsblå period från mitten av 90-talet… Ser fram emot att hälsa på dig i Stockholm och gå på Garbos. **Svempa**, principfast som få och med ett danssteg som får Travolta (och Frostis) att blekna. Synd att vi inte kan träffas lika ofta sedan du flyttade till Norwich. **David ”Jörgen Persson” Eriksson.** Skellefteås varmaste hjärta och klenaste rygg. Med dig är det ett nöje att vara Samsonov. Lite lätt avis på ditt bollsinne och att du träffat Ray Brown. **Magnus**, min snabbspringande vän i röda laget. Saknar söndagskvällarna med kokkaffe på Nydala. **Hasse Hansson**, för att du är en av få som förstår värdet av steroidhormonreceptorer och alltid har ett sanningens ord på lut. Tack även för alla roliga Stockholmskvällar. **Erik** som tappat lite av sitt sköna blekingemål, men är sig lik i övrigt. Räknar med en guidad tur i dykvattnet när jag väl skaffat cert. **Gussing**, bästa skånepågen i Ume. Inget slår att få fler fiskar än du. Skulle vara en snabbare tid på milen isf… ☺ **Christina**, som alltid vill hitta på något ”kulturellt” och bjuder på smarriga middagar i Röbäck. När får vi svänga våra lurviga till klezmer igen?

Ni som tillkommit under åren är förstås lika högt värderade.
Charlotte, principfast som Sven; sällskapsspel handlar om att utse en rättmätig vinnare och är inte ett sätt att ha trevligt tillsammans (sånt gillar en Jeopardymästare!). Hoppas du snart återvänder till Tunnelbacken. **Sara**. Tack för alla gastronomiska höjdpunkter. Middagarna hos dig var en ren lyx; ända tills vi prompt skulle spela det där gediget genomtråkiga kortspelet, vars namn jag tappat bort… **Sofia**. Tack för att du alltid ställer upp och är den snällaste tjejen på Tunnelbacken. Roligt att vi nu bor i samma kvarter! Flitiga, flitiga **Jeanette** som sannolikt skulle glömma sig själv på bussen om det vore möjligt. Tack för otaliga middagar på Thaiköket och Björken då vi skrivit varsin bok. **Håkan ”Bishops-Ja gärna” Hallberg.** Tack för alla trevliga fredagskvällar på sistone. Finlands sak är vår. ☺ **Linda**, för att du alltid är uppåt och håller Bossman på mattan när det behövs. Vi syns vid nästa reprisering av Krönikan. **Martina**, den londonska matkonnässören (snart i Stockholm; grattis till nya jobbet!). Hoppas få träffa dig och Magnus mer frekvent framöver. **Kicki** för att du vet allt om Umeå med omnejd och gärna diskuterar det med mig. **Anna** formerly known as **Anna Jansson,** en i den exklusiva skara som uppskattar jazz i tillräcklig utsträckning.

Niklas, vars tävlingssinne gör löprundorna ytterst konditionskrävande. Tack för det. **Maria E** för din sköna humor och att du tar med dig Gussing till Ume emellanåt. **Maria J** för gott grannskap. Ser fram emot ett möte på centercourten.

Tack också **Tobias, Henke, Natasha, Petra** & **Tom, Petra** & **Anders;** det är alltid kul när vi ses.

Tack också till korregänget för att vi har lika roligt än idag.
Micke J, vars musikkunskaper håller Nileskärklass (nåja, nästan…) och som vaknar mitt i natten för att berätta reseminnen. **Helena,** språkvetaren som kan fler tyska hälsningsfraser än Angela Merckel. Tack för expertråd kring bruket av gemener i doktorsavhandlingar. **Carol,** västkustens partypingla som vi hoppas få se i Umeå oftare framöver. **Micke B och Hanna**, som vet vilka lunchrestauranger man ska undvika i Umeå…

En stor eloge går till soppsällskaparna som bjudit på många gastronomiska upplevelser de senaste åren.
Micke W, indiepopparen som blev kansliråd och är lika imponerad av Anders Borgs insatser på jobbet som på gymmet. Tack för härliga diskussioner om allehanda aktualiteter. **Sarah,** vars soppor imponerar varje gång. **Fredik** och **Lina**. Era berättelser från småskolans och ”storskolans” värld är alltid lika underhållande.

Tack **Daniel** för att du som barndomsvän inte bangar för nostalgiska utflykter till Bowlinghallen och Domusrestaurangen och vet att påpeka att det finns en värld utanför universitetet. Dessutom är det uppskattat att du vet allt om världens samlade elektronikpark. Tack **Fredrik** and **Anders** för stenhårda plumpmatcher och roliga episoder i de mindre frikyrkliga delarna av Jönköping.

Tack **Mamma** och **Pappa** för att ni stöder mig fullt ut.

Tack **Jenny** och **Ayesha;** det är alltid lika mysigt att komma ned till er i Göteborg. Tack Jenny för att du säger till mig att träna lite mer och jobba lite mindre (och för referenskoll!).

REFERENCES

Adams NR 1995 Detection of the effects of phytoestrogens on sheep and cattle. *J Anim Sci* **73** 1509-1515.

Adams RG, Harrison JF & Scott P 1969 The development of cadmium-induced proteinuria, impaired renal function, and osteomalacia in alkaline battery workers. *Q J Med* **38** 425-443.

Adamsson E, Piscator M & Nogawa K 1979 Pulmonary and gastrointestinal exposure to cadmium oxide dust in a battery factory. *Environ Health Perspect* **28** 219-222.

Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T & Okada H 1991 Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am J Clin Nutr* **54** 1093-1100.

Adlercreutz H, Fotsis T, Watanabe S, Lampe J, Wahala K, Makela T & Hase T 1994 Determination of lignans and isoflavonoids in plasma by isotope dilution gas chromatography-mass spectrometry. *Cancer Detect Prev* **18** 259-271.

Ahtiainen P, Rulli S, Pakarainen T, Zhang FP, Poutanen M & Huhtaniemi I 2007 Phenotypic characterisation of mice with exaggerated and missing LH/hCG action. *Mol Cell Endocrinol* **260-262** 255-263.

Akesson A, Julin B & Wolk A 2008 Long-term dietary cadmium intake and postmenopausal endometrial cancer incidence: a population-based prospective cohort study. *Cancer Res* **68** 6435-6441.

Akesson A, Berglund M, Schutz A, Bjellerup P, Bremme K & Vahter M 2002 Cadmium exposure in pregnancy and lactation in relation to iron status. *Am J Public Health* **92** 284-287.

Almstrup K, Fernandez MF, Petersen JH, Olea N, Skakkebaek NE & Leffers H 2002 Dual effects of phytoestrogens result in u-shaped dose-response curves. *Environ Health Perspect* **110** 743-748.

Aluru N & Vijayan MM 2006 Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting steps in steroidogenesis. *Endocrinology* **147** 1895-1903.

Anakwe OO & Moger WH 1984 Ontogeny of rodent testicular androgen production in response to isoproterenol and luteinizing hormone in vitro. *Biol Reprod* **30** 1142-1152.

Anakwe OO, Murphy PR & Moger WH 1985 Characterization of beta-adrenergic binding sites on rodent Leydig cells. *Biol Reprod* **33** 815-826.

Andersen O, Nielsen JB, Sorensen JA & Scherrebeck L 1994 Experimental localization of intestinal uptake sites for metals (Cd, Hg, Zn, Se) in vivo in mice. *Environ Health Perspect* **102 Suppl 3** 199-206.

Andersson AM, Jorgensen N, Main KM, Toppari J, Rajpert-De Meyts E, Leffers H, Juul A, Jensen TK & Skakkebaek NE 2008 Adverse trends in male reproductive health: we may have reached a crucial 'tipping point'. *Int J Androl* **31** 74-80.

Andrade AJ, Grande SW, Talsness CE, Grote K, Golombiewski A, Sterner-Kock A & Chahoud I 2006a A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): effects on androgenic status, developmental landmarks and testicular histology in male offspring rats. *Toxicology* **225** 64-74.

Andrade AJ, Grande SW, Talsness CE, Gericke C, Grote K, Golombiewski A, Sterner-Kock A & Chahoud I 2006b A dose response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult male offspring rats. *Toxicology* **228** 85-97.

Antony FF, Aruldhas MM, Udhayakumar RC, Maran RR & Govindarajulu P 1995 Inhibition of Leydig cell activity in vivo and in vitro in hypothyroid rats. *J Endocrinol* **144** 293-300.

Anway MD, Cupp AS, Uzumcu M & Skinner MK 2005 Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308** 1466-1469.

Atkinson C, Frankenfeld CL & Lampe JW 2005 Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med (Maywood)* **230** 155-170.

ATSDR 1999 Toxicological profile for cadmium. Agency for Toxic Substances and Disease Registry. Atlanta, GA.

Auger J, Kunstmann JM, Czyglik F & Jouannet P 1995 Decline in semen quality among fertile men in Paris during the past 20 years. *N Engl J Med* **332** 281-285.

Bagchi D, Bagchi M, Tang L & Stohs SJ 1997 Comparative in vitro and in vivo protein kinase C activation by selected pesticides and transition metal salts. *Toxicol Lett* **91** 31-37.

Barregard L, Svalander C, Schutz A, Westberg G, Sallsten G, Blohme I, Molne J, Attman PO & Haglind P 1999 Cadmium, mercury, and lead in kidney cortex of the general Swedish population: a study of biopsies from living kidney donors. *Environ Health Perspect* **107** 867-871.

Beck V, Rohr U & Jungbauer A 2005 Phytoestrogens derived from red clover: an alternative to estrogen replacement therapy? *J Steroid Biochem Mol Biol* **94** 499-518.

Becker K, Kaus S, Krause C, Lepom P, Schulz C, Seiwert M & Seifert B 2002 German Environmental Survey 1998 (GerES III): environmental pollutants in blood of the German population. *Int J Hyg Environ Health* **205** 297-308.

Becker K, Seiwert M, Angerer J, Heger W, Koch HM, Nagorka R, Rosskamp E, Schluter C, Seifert B & Ullrich D 2004 DEHP metabolites in urine of children and DEHP in house dust. *Int J Hyg Environ Health* **207** 409-417.

Bemanian V, Male R & Goksoyr A 2004 The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR- and ERalpha-signalling pathways. *Comp Hepatol* **3** 2.

Bench G, Corzett MH, Martinelli R & Balhorn R 1999 Cadmium concentrations in the testes, sperm, and spermatids of mice subjected to long-term cadmium chloride exposure. *Cytometry* **35** 30-36.

Bennets HW, Underwood EJ & Shier FL 1946 A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Aust. Vet. J.* **22** 2-12.

Berry MD & Boulton AA 2000 Glyceraldehyde-3-phosphate dehydrogenase and apoptosis. *J Neurosci Res* **60** 150-154.
Bianco JJ, McPherson SJ, Wang H, Prins GS & Risbridger GP 2006 Transient neonatal estrogen exposure to estrogen-deficient mice (aromatase knockout) reduces prostate weight and induces inflammation in late life. *Am J Pathol* **168** 1869-1878.
Biswas NM, Sen Gupta R, Chattopadhyay A, Choudhury GR & Sarkar M 2001 Effect of atenolol on cadmium-induced testicular toxicity in male rats. *Reprod Toxicol* **15** 699-704.
Bitman J, Cecil HC, Harris SJ & Fries GF 1969 DDT induces a decrease in eggshell calcium. *Nature* **224** 44-46.
Bjurulf E & Selstam G 1996 Rat luteinizing hormone receptor messenger ribonucleic acid expression and luteolysis: inhibition by prostaglandin F2 alpha. *Biol Reprod* **54** 1350-1355.
Block C, Freyermuth S, Beyersmann D & Malviya AN 1992 Role of cadmium in activating nuclear protein kinase C and the enzyme binding to nuclear protein. *J Biol Chem* **267** 19824-19828.
Bogatcheva NV, Ferlin A, Feng S, Truong A, Gianesello L, Foresta C & Agoulnik AI 2007 T222P mutation of the insulin-like 3 hormone receptor LGR8 is associated with testicular maldescent and hinders receptor expression on the cell surface membrane. *Am J Physiol Endocrinol Metab* **292** E138-144.
Boisen KA, Chellakooty M, Schmidt IM, Kai CM, Damgaard IN, Suomi AM, Toppari J, Skakkebaek NE & Main KM 2005 Hypospadias in a cohort of 1072 Danish newborn boys: prevalence and relationship to placental weight, anthropometrical measurements at birth, and reproductive hormone levels at three months of age. *J Clin Endocrinol Metab* **90** 4041-4046.
Boisen KA, Kaleva M, Main KM, Virtanen HE, Haavisto AM, Schmidt IM, Chellakooty M, Damgaard IN, Mau C, Reunanen M, Skakkebaek NE & Toppari J 2004 Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. *Lancet* **363** 1264-1269.
Bomhard E, Vogel O & Loser E 1987 Chronic effects on single and multiple oral and subcutaneous cadmium administrations on the testes of Wistar rats. *Cancer Lett* **36** 307-315.
Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Henriksen TB, Scheike T, Giwercman A, Olsen J & Skakkebaek NE 1998 Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* **352** 1172-1177.
Bornman MS, Pretorius E, Marx J, Smit E & van der Merwe CF 2007 Ultrastructural effects of DDT, DDD, and DDE on neural cells of the chicken embryo model. *Environ Toxicol* **22** 328-336.
Bose HS, Baldwin MA & Miller WL 1998 Incorrect folding of steroidogenic acute regulatory protein (StAR) in congenital lipoid adrenal hyperplasia. *Biochemistry* **37** 9768-9775.
Boukari K, Ciampi ML, Guiochon-Mantel A, Young J, Lombes M & Meduri G 2007 Human fetal testis: source of estrogen and target of estrogen action. *Hum Reprod* **22** 1885-1892.

Bovee TF, Helsdingen RJ, Rietjens IM, Keijer J & Hoogenboom RL 2004 Rapid yeast estrogen bioassays stably expressing human estrogen receptors alpha and beta, and green fluorescent protein: a comparison of different compounds with both receptor types. *J Steroid Biochem Mol Biol* **91** 99-109.
Bray TM & Bettger WJ 1990 The physiological role of zinc as an antioxidant. *Free Radic Biol Med* **8** 281-291.
Bruce B, Messina M & Spiller GA 2003 Isoflavone supplements do not affect thyroid function in iodine-replete postmenopausal women. *J Med Food* **6** 309-316.
Brzoska MM & Moniuszko-Jakoniuk J 2004 Low-level exposure to cadmium during the lifetime increases the risk of osteoporosis and fractures of the lumbar spine in the elderly: studies on a rat model of human environmental exposure. *Toxicol Sci* **82** 468-477.
Burlington H & Lindeman VF 1950 Effect of DDT on testes and secondary sex characters of white leghorn cockerels. *Proc Soc Exp Biol Med* **74** 48-51.
Calafat AM, Slakman AR, Silva MJ, Herbert AR & Needham LL 2004 Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites. *J Chromatogr B Analyt Technol Biomed Life Sci* **805** 49-56.
Carlsen E, Giwercman A, Keiding N & Skakkebaek NE 1992 Evidence for decreasing quality of semen during past 50 years. *Bmj* **305** 609-613.
Carmeci C, Thompson DA, Ring HZ, Francke U & Weigel RJ 1997 Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics* **45** 607-617.
Carreau S, Genissel C, Bilinska B & Levallet J 1999 Sources of oestrogen in the testis and reproductive tract of the male. *Int J Androl* **22** 211-223.
Castellano JM, Navarro VM, Fernandez-Fernandez R, Castano JP, Malagon MM, Aguilar E, Dieguez C, Magni P, Pinilla L & Tena-Sempere M 2006 Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. *Mol Cell Endocrinol* **257-258** 75-83.
Cavaliere C, Cucci F, Foglia P, Guarino C, Samperi R & Lagana A 2007 Flavonoid profile in soybeans by high-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* **21** 2177-2187.
Chan HM & Cherian MG 1992 Protective roles of metallothionein and glutathione in hepatotoxicity of cadmium. *Toxicology* **72** 281-290.
Chan HM, Tamura Y, Cherian MG & Goyer RA 1993 Pregnancy-associated changes in plasma metallothionein concentration and renal cadmium accumulation in rats. *Proc Soc Exp Biol Med* **202** 420-427.
Chang HC & Doerge DR 2000 Dietary genistein inactivates rat thyroid peroxidase in vivo without an apparent hypothyroid effect. *Toxicol Appl Pharmacol* **168** 244-252.
Cherian MG 1979 Metabolism of orally administered cadmium-metallothionein in mice. *Environ Health Perspect* **28** 127-130.
Chernoff N 1973 Teratogenic effects of cadmium in rats. *Teratology* **8** 29-32.
Chilvers C, Pike MC, Forman D, Fogelman K & Wadsworth ME 1984 Apparent doubling of frequency of undescended testis in England and Wales in 1962-81. *Lancet* **2** 330-332.

Choi JS, Kim KR, Ahn DW & Park YS 1999 Cadmium inhibits albumin endocytosis in opossum kidney epithelial cells. *Toxicol Appl Pharmacol* **161** 146-152.
Chung PH, Sandhoff TW & McLean MP 1998 Hormone and prostaglandin F2 alpha regulation of messenger ribonucleic acid encoding steroidogenic acute regulatory protein in human corpora lutea. *Endocrine* **8** 153-160.
Clarke DB & Lloyd AS 2004 Dietary exposure estimates of isoflavones from the 1998 UK Total Diet Study. *Food Addit Contam* **21** 305-316.
Cline JM, Franke AA, Register TC, Golden DL & Adams MR 2004 Effects of dietary isoflavone aglycones on the reproductive tract of male and female mice. *Toxicol Pathol* **32** 91-99.
Collins BM, McLachlan JA & Arnold SF 1997 The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids* **62** 365-372.
Colon I, Caro D, Bourdony CJ & Rosario O 2000 Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. *Environ Health Perspect* **108** 895-900.
Conrad ME & Umbreit JN 2000 Iron absorption and transport-an update. *Am J Hematol* **64** 287-298.
Cooper RL, Goldman JM, Rehnberg GL, McElroy WK & Hein JF 1987 Effects of metal cations on pituitary hormone secretion in vitro. *J Biochem Toxicol* **2** 241-249.
Cooper RL, Stoker TE, Tyrey L, Goldman JM & McElroy WK 2000 Atrazine disrupts the hypothalamic control of pituitary-ovarian function. *Toxicol Sci* **53** 297-307.
Couse JF, Yates MM, Deroo BJ & Korach KS 2005 Estrogen receptor-beta is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. *Endocrinology* **146** 3247-3262.
Coyle P, Philcox JC, Carey LC & Rofe AM 2002 Metallothionein: the multipurpose protein. *Cell Mol Life Sci* **59** 627-647.
Damber JE, Bergh A, Selstam G & Södergård R 1983 Estrogen Receptor and Aromatase Activity in the Testes of Unilateral Cryptorchid Rat. *Arch Androl* **11** 259-263.
Danielsson BR, Dencker L, Lindgren A & Tjalve H 1984 Accumulation of toxic metals in male reproduction organs. *Arch Toxicol Suppl* **7** 177-180.
Davis BJ, Weaver R, Gaines LJ & Heindel JJ 1994 Mono-(2-ethylhexyl) phthalate suppresses estradiol production independent of FSH-cAMP stimulation in rat granulosa cells. *Toxicol Appl Pharmacol* **128** 224-228.
De Smet H, Blust R & Moens L 2001 Cadmium-binding to transferrin in the plasma of the common carp Cyprinus carpio. *Comp Biochem Physiol C Toxicol Pharmacol* **128** 45-53.
Delclos KB, Bucci TJ, Lomax LG, Latendresse JR, Warbritton A, Weis CC & Newbold RR 2001 Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. *Reprod Toxicol* **15** 647-663.
Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ & Wiltbank MC 2002 Regulation of progesterone and prostaglandin F2alpha production in the CL. *Mol Cell Endocrinol* **191** 65-80.

Didolkar AK, Gurjar A, Joshi UM, Sheth AR & Roychowdhury D 1981 Effect of prostaglandins A-1, E-2 and F-2 alpha on blood plasma levels of testosterone, LH and FSH in male rats. *Andrologia* **13** 50-55.
Dieckmann KP & Pichlmeier U 2004 Clinical epidemiology of testicular germ cell tumors. *World J Urol* **22** 2-14.
Dillingham BL, McVeigh BL, Lampe JW & Duncan AM 2007 Soy protein isolates of varied isoflavone content do not influence serum thyroid hormones in healthy young men. *Thyroid* **17** 131-137.
Doerge DR & Sheehan DM 2002 Goitrogenic and estrogenic activity of soy isoflavones. *Environ Health Perspect* **110 Suppl 3** 349-353.
El-Demerdash FM, Yousef MI, Kedwany FS & Baghdadi HH 2004 Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective role of vitamin E and beta-carotene. *Food Chem Toxicol* **42** 1563-1571.
El-Gehani F, Zhang FP, Pakarinen P, Rannikko A & Huhtaniemi I 1998 Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. *Biol Reprod* **58** 116-123.
Elinder CG, Friberg L, Lind B & Jawaid M 1983a Lead and cadmium levels in blood samples from the general population of Sweden. *Environ Res* **30** 233-253.
Elinder CG, Kjellstrom T, Lind B, Linnman L, Piscator M & Sundstedt K 1983b Cadmium exposure from smoking cigarettes: variations with time and country where purchased. *Environ Res* **32** 220-227.
Elsisi AE, Carter DE & Sipes IG 1989 Dermal absorption of phthalate diesters in rats. *Fundam Appl Toxicol* **12** 70-77.
Epner DE, Sawa A & Isaacs JT 1999 Glyceraldehyde-3-phosphate dehydrogenase expression during apoptosis and proliferation of rat ventral prostate. *Biol Reprod* **61** 687-691.
Erfurt C, Roussa E & Thevenod F 2003 Apoptosis by Cd2+ or CdMT in proximal tubule cells: different uptake routes and permissive role of endo/lysosomal CdMT uptake. *Am J Physiol Cell Physiol* **285** C1367-1376.
Erkkila K, Henriksen K, Hirvonen V, Rannikko S, Salo J, Parvinen M & Dunkel L 1997 Testosterone regulates apoptosis in adult human seminiferous tubules in vitro. *J Clin Endocrinol Metab* **82** 2314-2321.
Faber KA & Hughes CL, Jr. 1991 The effect of neonatal exposure to diethylstilbestrol, genistein, and zearalenone on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat. *Biol Reprod* **45** 649-653.
Feige JN, Gelman L, Rossi D, Zoete V, Metivier R, Tudor C, Anghel SI, Grosdidier A, Lathion C, Engelborghs Y, Michielin O, Wahli W & Desvergne B 2007 The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor gamma modulator that promotes adipogenesis. *J Biol Chem* **282** 19152-19166.
Fennell TR, Krol WL, Sumner SC & Snyder RW 2004 Pharmacokinetics of dibutylphthalate in pregnant rats. *Toxicol Sci* **82** 407-418.

Ferlin A, Bogatcheva NV, Gianesello L, Pepe A, Vinanzi C, Agoulnik AI & Foresta C 2006 Insulin-like factor 3 gene mutations in testicular dysgenesis syndrome: clinical and functional characterization. *Mol Hum Reprod* **12** 401-406.
Fernandez EL, Gustafson AL, Andersson M, Hellman B & Dencker L 2003 Cadmium-induced changes in apoptotic gene expression levels and DNA damage in mouse embryos are blocked by zinc. *Toxicol Sci* **76** 162-170.
Fiedler EP, Plouffe L, Jr., Hales DB, Hales KH & Khan I 1999 Prostaglandin F(2alpha) induces a rapid decline in progesterone production and steroidogenic acute regulatory protein expression in isolated rat corpus luteum without altering messenger ribonucleic acid expression. *Biol Reprod* **61** 643-650.
Fisher JS, Macpherson S, Marchetti N & Sharpe RM 2003 Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Hum Reprod* **18** 1383-1394.
Flanagan PR, McLellan JS, Haist J, Cherian G, Chamberlain MJ & Valberg LS 1978 Increased dietary cadmium absorption in mice and human subjects with iron deficiency. *Gastroenterology* **74** 841-846.
Fletcher RJ 2003 Food sources of phyto-oestrogens and their precursors in Europe. *Br J Nutr* **89 Suppl 1** S39-43.
Fluck CE, Miller WL & Auchus RJ 2003 The 17, 20-lyase activity of cytochrome p450c17 from human fetal testis favors the delta5 steroidogenic pathway. *J Clin Endocrinol Metab* **88** 3762-3766.
Foster PM 2006 Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int J Androl* **29** 140-147; discussion 181-145.
Foster PM, Cattley RC & Mylchreest E 2000 Effects of di-n-butyl phthalate (DBP) on male reproductive development in the rat: implications for human risk assessment. *Food Chem Toxicol* **38** S97-99.
Fowler PA, Abramovich DR, Haites NE, Cash P, Groome NP, Al-Qahtani A, Murray TJ & Lea RG 2007 Human fetal testis Leydig cell disruption by exposure to the pesticide dieldrin at low concentrations. *Hum Reprod* **22** 2919-2927.
Frankenfeld CL, Atkinson C, Thomas WK, Gonzalez A, Jokela T, Wahala K, Schwartz SM, Li SS & Lampe JW 2005 High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart. *Br J Nutr* **94** 873-876.
Frederiksen H, Skakkebaek NE & Andersson AM 2007 Metabolism of phthalates in humans. *Mol Nutr Food Res* **51** 899-911.
Friberg L 1950 Health hazards in the manufacture of alkaline accumulators with special reference to chronic cadmium poisoning; a clinical and experimental study. *Acta Med Scand Suppl* **240** 1-124.
Friberg L 1974 Cadmium in the environment. Cleveland: CRC Press.
Froment P, Fabre S, Dupont J, Pisselet C, Chesneau D, Staels B & Monget P 2003 Expression and functional role of peroxisome proliferator-activated receptor-gamma in ovarian folliculogenesis in the sheep. *Biol Reprod* **69** 1665-1674.
Frungieri MB, Gonzalez-Calvar SI, Parborell F, Albrecht M, Mayerhofer A & Calandra RS 2006 Cyclooxygenase-2 and prostaglandin F2 alpha in Syrian hamster

Leydig cells: Inhibitory role on luteinizing hormone/human chorionic gonadotropin-stimulated testosterone production. *Endocrinology* **147** 4476-4485.
Fukuzawa NH, Ohsako S, Wu Q, Sakaue M, Fujii-Kuriyama Y, Baba T & Tohyama C 2004 Testicular cytochrome P450scc and LHR as possible targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the mouse. *Mol Cell Endocrinol* **221** 87-96.
Gal M, Orly J, Barr I, Algur N, Boldes R & Diamant YZ 1994 Low dose ketoconazole attenuates serum androgen levels in patients with polycystic ovary syndrome and inhibits ovarian steroidogenesis in vitro. *Fertil Steril* **61** 823-832.
Gallentine ML, Morey AF & Thompson IM, Jr. 2001 Hypospadias: a contemporary epidemiologic assessment. *Urology* **57** 788-790.
Gardner-Thorpe D, O'Hagen C, Young I & Lewis SJ 2003 Dietary supplements of soya flour lower serum testosterone concentrations and improve markers of oxidative stress in men. *Eur J Clin Nutr* **57** 100-106.
Gaskell TL, Robinson LL, Groome NP, Anderson RA & Saunders PT 2003 Differential expression of two estrogen receptor beta isoforms in the human fetal testis during the second trimester of pregnancy. *J Clin Endocrinol Metab* **88** 424-432.
Ge RS, Chen GR, Dong Q, Akingbemi B, Sottas CM, Santos M, Sealfon SC, Bernard DJ & Hardy MP 2007 Biphasic effects of postnatal exposure to diethylhexylphthalate on the timing of puberty in male rats. *J Androl* **28** 513-520.
Giusti RM, Iwamoto K & Hatch EE 1995 Diethylstilbestrol revisited: a review of the long-term health effects. *Ann Intern Med* **122** 778-788.
Giwercman A, Rylander L, Rignell-Hydbom A, Jonsson BA, Pedersen HS, Ludwicki JK, Lesovoy V, Zvyezday V, Spano M, Manicardi GC, Bizzaro D, Bonefeld-Jorgensen EC, Toft G, Bonde JP, Giwercman C *et al.* 2007 Androgen receptor gene CAG repeat length as a modifier of the association between persistent organohalogen pollutant exposure markers and semen characteristics. *Pharmacogenet Genomics* **17** 391-401.
Goff AK 2004 Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. *Biol Reprod* **71** 11-16.
Goyer RA, Epstein S, Bhattacharyya M, Korach KS & Pounds J 1994 Environmental risk factors for osteoporosis. *Environ Health Perspect* **102** 390-394.
Graven KK, McDonald RJ & Farber HW 1998 Hypoxic regulation of endothelial glyceraldehyde-3-phosphate dehydrogenase. *Am J Physiol* **274** C347-355.
Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E & Guillette L 2001 Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* **7** 248-264.
Gray LE, Wilson V, Stoker T, Lambright C, Furr J, Noriega N, Hartig P, Cardon M, Rosen M, Ankley G, Hotchkiss A, Orlando EF, Guillette LJ & Kelce WR 2005 Environmental Androgens and Antiandrogens: An Expanding Chemical Universe. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, edn Second. Ed. RK Naz. Boca Raton: CRC Press.

Gray LE, Jr., Ostby J, Furr J, Price M, Veeramachaneni DN & Parks L 2000 Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci* **58** 350-365.

Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL & Ostby J 1999 Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* **15** 94-118.

Groten JP, Sinkeldam EJ, Luten JB & van Bladeren PJ 1991 Cadmium accumulation and metallothionein concentrations after 4-week dietary exposure to cadmium chloride or cadmium-metallothionein in rats. *Toxicol Appl Pharmacol* **111** 504-513.

Guillette LJ, Jr. 2006 Endocrine disrupting contaminants--beyond the dogma. *Environ Health Perspect* **114 Suppl 1** 9-12.

Gunn SA, Gould TC & Anderson WA 1968 Selectivity of organ response to cadmium injury and various protective measures. *J Pathol Bacteriol* **96** 89-96.

Habert R, Lejeune H & Saez JM 2001 Origin, differentiation and regulation of fetal and adult Leydig cells. *Mol Cell Endocrinol* **179** 47-74.

Haider SG 2004 Cell biology of Leydig cells in the testis. *Int Rev Cytol* **233** 181-241.

Han S, Ritzenthaler JD, Rivera HN & Roman J 2005 Peroxisome proliferator-activated receptor-gamma ligands suppress fibronectin gene expression in human lung carcinoma cells: involvement of both CRE and Sp1. *Am J Physiol Lung Cell Mol Physiol* **289** L419-428.

Harada N, Yasunaga R, Higashimura Y, Yamaji R, Fujimoto K, Moss J, Inui H & Nakano Y 2007 Glyceraldehyde-3-phosphate dehydrogenase enhances transcriptional activity of androgen receptor in prostate cancer cells. *J Biol Chem* **282** 22651-22661.

Harper PA, Wong JY, Lam MS & Okey AB 2002 Polymorphisms in the human AH receptor. *Chem Biol Interact* **141** 161-187.

Harris CA, Henttu P, Parker MG & Sumpter JP 1997 The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* **105** 802-811.

Hauser R & Calafat AM 2005 Phthalates and human health. *Occup Environ Med* **62** 806-818.

Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S & Calafat AM 2007 DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Hum Reprod* **22** 688-695.

Hazell AS, Desjardins P & Butterworth RF 1999 Increased expression of glyceraldehyde-3-phosphate dehydrogenase in cultured astrocytes following exposure to manganese. *Neurochem Int* **35** 11-17.

Heinonen S, Wahala K & Adlercreutz H 1999 Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6'-OH-O-dma, and cis-4-OH-equol in human urine by gas chromatography-mass spectroscopy using authentic reference compounds. *Anal Biochem* **274** 211-219.

Hellstrom L, Elinder CG, Dahlberg B, Lundberg M, Jarup L, Persson B & Axelson O 2001 Cadmium exposure and end-stage renal disease. *Am J Kidney Dis* **38** 1001-1008.
Herbst AL, Ulfelder H & Poskanzer DC 1971 Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* **284** 878-881.
Heudorf U, Mersch-Sundermann V & Angerer J 2007 Phthalates: toxicology and exposure. *Int J Hyg Environ Health* **210** 623-634.
Hew KW, Ericson WA & Welsh MJ 1993 A single low cadmium dose causes failure of spermiation in the rat. *Toxicol Appl Pharmacol* **121** 15-21.
Hogberg J, Hanberg A, Berglund M, Skerfving S, Remberger M, Calafat AM, Filipsson AF, Jansson B, Johansson N, Appelgren M & Hakansson H 2008 Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. *Environ Health Perspect* **116** 334-339.
Holm L, Blomqvist A, Brandt I, Brunstrom B, Ridderstrale Y & Berg C 2006 Embryonic exposure to o,p'-DDT causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. *Environ Toxicol Chem* **25** 2787-2793.
Howdeshell KL, Wilson VS, Furr J, Lambright CR, Rider CV, Blystone CR, Hotchkiss AK & Gray LE, Jr. 2008 A mixture of five phthalate esters inhibits fetal testicular testosterone production in the sprague-dawley rat in a cumulative, dose-additive manner. *Toxicol Sci* **105** 153-165.
Huang BM & Liu MY 2004 Inhibitory actions of lead on steroidogenesis in MA-10 mouse Leydig tumor cells. *Arch Androl* **50** 5-9.
Huang PC, Kuo PL, Guo YL, Liao PC & Lee CC 2007 Associations between urinary phthalate monoesters and thyroid hormones in pregnant women. *Hum Reprod* **22** 2715-2722.
Huel G, Boudene C & Ibrahim MA 1981 Cadmium and lead content of maternal and newborn hair: relationship to parity, birth weight, and hypertension. *Arch Environ Health* **36** 221-227.
Hur H & Rafii F 2000 Biotransformation of the isoflavonoids biochanin A, formononetin, and glycitein by Eubacterium limosum. *FEMS Microbiol Lett* **192** 21-25.
Huyghe E, Matsuda T & Thonneau P 2003 Increasing incidence of testicular cancer worldwide: a review. *J Urol* **170** 5-11.
IARC 1992 Cadmium in the human environment: toxicity and carcinogenicity. IARC Scientific Publications. No. 118. International Agency for Research on Cancer (WHO), Lyon, France.
IPCS 2002 The International Programme on Chemical Safety (IPCS): Global assessment of the state-of-the-science of endocrine disruptors. WHO/CPS/EDC/02.2.
Ishitobi H, Mori K, Yoshida K & Watanabe C 2007 Effects of perinatal exposure to low-dose cadmium on thyroid hormone-related and sex hormone receptor gene expressions in brain of offspring. *Neurotoxicology* **28** 790-797.

Ito Y, Yokota H, Wang R, Yamanoshita O, Ichihara G, Wang H, Kurata Y, Takagi K & Nakajima T 2005 Species differences in the metabolism of di(2-ethylhexyl) phthalate (DEHP) in several organs of mice, rats, and marmosets. *Arch Toxicol* **79** 147-154.

Jaakkola JJ & Knight TL 2008 The Role of Exposure to Phthalates from Polyvinyl Chloride Products in the Development of Asthma and Allergies: A Systematic Review and Meta-analysis. *Environ Health Perspect* **116** 845-853.

Jakimiuk AJ, Weitsman SR, Yen HW, Bogusiewicz M & Magoffin DA 2002 Estrogen receptor alpha and beta expression in theca and granulosa cells from women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **87** 5532-5538.

Jana NR, Halder S & Bhattacharya S 1996 Thyroid hormone induces a 52 kDa soluble protein in goat testis Leydig cell which stimulates androgen release. *Biochim Biophys Acta* **1292** 209-214.

Janjua NR, Frederiksen H, Skakkebaek NE, Wulf HC & Andersson AM 2008 Urinary excretion of phthalates and paraben after repeated whole-body topical application in humans. *Int J Androl* **31** 118-130.

Jarup L 2002 Cadmium overload and toxicity. *Nephrol Dial Transplant* **17 Suppl 2** 35-39.

Jarup L 2003 Hazards of heavy metal contamination. *Br Med Bull* **68** 167-182.

Jarup L & Elinder CG 1994 Dose-response relations between urinary cadmium and tubular proteinuria in cadmium-exposed workers. *Am J Ind Med* **26** 759-769.

Jarup L, Berglund M, Elinder CG, Nordberg G & Vahter M 1998 Health effects of cadmium exposure--a review of the literature and a risk estimate. *Scand J Work Environ Health* **24 Suppl 1** 1-51.

Jarup L, Hellstrom L, Alfven T, Carlsson MD, Grubb A, Persson B, Pettersson C, Spang G, Schutz A & Elinder CG 2000 Low level exposure to cadmium and early kidney damage: the OSCAR study. *Occup Environ Med* **57** 668-672.

Jin T, Nordberg G, Ye T, Bo M, Wang H, Zhu G, Kong Q & Bernard A 2004 Osteoporosis and renal dysfunction in a general population exposed to cadmium in China. *Environ Res* **96** 353-359.

Jobling S, Reynolds T, White R, Parker MG & Sumpter JP 1995 A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* **103** 582-587.

Joensen UN, Jorgensen N, Rajpert-De Meyts E & Skakkebaek NE 2008 Testicular dysgenesis syndrome and Leydig cell function. *Basic Clin Pharmacol Toxicol* **102** 155-161.

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, Clarke R, Sholler PF, Lirio AA, Foss C, Reiter R, Trock B, Paik S & Martin MB 2003 Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. *Nat Med* **9** 1081-1084.

Juengel JL, Haworth JD, Rollyson MK, Silva PJ, Sawyer HR & Niswender GD 2000 Effect of dose of prostaglandin F(2alpha) on steroidogenic components and oligonucleosomes in ovine luteal tissue. *Biol Reprod* **62** 1047-1051.

Jurasovic J, Cvitkovic P, Pizent A, Colak B & Telisman S 2004 Semen quality and reproductive endocrine function with regard to blood cadmium in Croatian male subjects. *Biometals* **17** 735-743.
Kalcher K, Kern W & Pietsch R 1993 Cadmium and lead in the smoke of a filter cigarette. *Sci Total Environ* **128** 21-35.
Kato K, Silva MJ, Reidy JA, Hurtz D, 3rd, Malek NA, Needham LL, Nakazawa H, Barr DB & Calafat AM 2004 Mono(2-ethyl-5-hydroxyhexyl) phthalate and mono-(2-ethyl-5-oxohexyl) phthalate as biomarkers for human exposure assessment to di-(2-ethylhexyl) phthalate. *Environ Health Perspect* **112** 327-330.
Keck C, Bramkamp G, Behre HM, Muller C, Jockenhovel F & Nieschlag E 1995 Lack of correlation between cadmium in seminal plasma and fertility status of nonexposed individuals and two cadmium-exposed patients. *Reprod Toxicol* **9** 35-40.
Kelly M 1988 Case reports of individuals with oligospermia and methylene chloride exposures. *Reprod Toxicol* **2** 13-17.
Khan MI, Rosberg S, Lahav M, Lamprecht SA, Selstam G, Herlitz H & Ahren K 1979 Studies on the mechanism of action of the inhibitory effect of prostaglandin F2 alpha on cyclic AMP accumulation in rat corpora lutea of various ages. *Biol Reprod* **21** 1175-1183.
Khan S, Khan MA, Bhatnagar D, Yadav P & Sarkar S 1991 Zinc protection against lipid peroxidation from cadmium. *Indian J Exp Biol* **29** 823-825.
Khan S, Barhoumi R, Burghardt R, Liu S, Kim K & Safe S 2006 Molecular mechanism of inhibitory aryl hydrocarbon receptor-estrogen receptor/Sp1 cross talk in breast cancer cells. *Mol Endocrinol* **20** 2199-2214.
Kim SC, Cho MK & Kim SG 2003 Cadmium-induced non-apoptotic cell death mediated by oxidative stress under the condition of sulfhydryl deficiency. *Toxicol Lett* **144** 325-336.
King LM, Banks WA & George WJ 1999 Differences in cadmium transport to the testis, epididymis, and brain in cadmium-sensitive and -resistant murine strains 129/J and A/J. *J Pharmacol Exp Ther* **289** 825-830.
King LM, Anderson MB, Sikka SC & George WJ 1998 Murine strain differences and the effects of zinc on cadmium concentrations in tissues after acute cadmium exposure. *Arch Toxicol* **72** 650-655.
Kjellstrom T & Nordberg GF 1978 A kinetic model of cadmium metabolism in the human being. *Environ Res* **16** 248-269.
Kluwe WM 1982 Overview of phthalate ester pharmacokinetics in mammalian species. *Environ Health Perspect* **45** 3-9.
Koch HM, Preuss R & Angerer J 2006 Di(2-ethylhexyl)phthalate (DEHP): human metabolism and internal exposure-- an update and latest results. *Int J Androl* **29** 155-165; discussion 181-155.
Kouki T, Kishitake M, Okamoto M, Oosuka I, Takebe M & Yamanouchi K 2003 Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: estrous cycle and lordosis. *Horm Behav* **44** 140-145.
Kruger T, Long M & Bonefeld-Jorgensen EC 2008 Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. *Toxicology* **246** 112-123.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B & Gustafsson JA 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139** 4252-4263.
Kurzer MS 2002 Hormonal effects of soy in premenopausal women and men. *J Nutr* **132** 570S-573S.
Kurzer MS & Xu X 1997 Dietary phytoestrogens. *Annu Rev Nutr* **17** 353-381.
Kusner LL, Sarthy VP & Mohr S 2004 Nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase: a role in high glucose-induced apoptosis in retinal Muller cells. *Invest Ophthalmol Vis Sci* **45** 1553-1561.
Lafuente A, Cano P & Esquifino A 2003 Are cadmium effects on plasma gonadotropins, prolactin, ACTH, GH and TSH levels, dose-dependent? *Biometals* **16** 243-250.
Lafuente A, Gonzalez-Carracedo A, Romero A, Cabaleiro T & Esquifino AI 2005 Toxic effects of cadmium on the regulatory mechanism of dopamine and serotonin on prolactin secretion in adult male rats. *Toxicol Lett* **155** 87-96.
Lague E & Tremblay JJ 2008 Antagonistic Effects of Testosterone and the Endocrine Disruptor Mehp on Insl3 Transcription in Leydig Cells. *Endocrinology*.
Lamartiniere CA, Wang J, Smith-Johnson M & Eltoum IE 2002 Daidzein: bioavailability, potential for reproductive toxicity, and breast cancer chemoprevention in female rats. *Toxicol Sci* **65** 228-238.
Lampe JW 2003 Isoflavonoid and lignan phytoestrogens as dietary biomarkers. *J Nutr* **133 Suppl 3** 956S-964S.
Lampe JW, Karr SC, Hutchins AM & Slavin JL 1998 Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med* **217** 335-339.
Lancranjan I, Popescu HI, O GA, Klepsch I & Serbanescu M 1975 Reproductive ability of workmen occupationally exposed to lead. *Arch Environ Health* **30** 396-401.
Lapinskas PJ, Brown S, Leesnitzer LM, Blanchard S, Swanson C, Cattley RC & Corton JC 2005 Role of PPARalpha in mediating the effects of phthalates and metabolites in the liver. *Toxicology* **207** 149-163.
Lashley S, Calafat A, Barr D, Ledoux T, Hore P, Lake M, Robson M & Smulian J 2004 Endocrine disruptors in the maternal and fetal compartments. *Am. J. Obstet. Gynecol.* **191** S140.
Laskey JW & Phelps PV 1991 Effect of cadmium and other metal cations on in vitro Leydig cell testosterone production. *Toxicol Appl Pharmacol* **108** 296-306.
Laskey JW, Rehnberg GL, Laws SC & Hein JF 1984 Reproductive effects of low acute doses of cadmium chloride in adult male rats. *Toxicol Appl Pharmacol* **73** 250-255.
Latini G, De Felice C, Presta G, Del Vecchio A, Paris I, Ruggieri F & Mazzeo P 2003 In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. *Environ Health Perspect* **111** 1783-1785.
Laws SC, Ferrell JM, Stoker TE, Schmid J & Cooper RL 2000 The effects of atrazine on female wistar rats: an evaluation of the protocol for assessing pubertal development and thyroid function. *Toxicol Sci* **58** 366-376.

Lazarevic B, Karlsen SJ & Saatcioglu F 2008 Genistein differentially modulates androgen-responsive gene expression and activates JNK in LNCaP cells. *Oncol Rep* **19** 1231-1235.

Le Jossic-Corcos C, Duclos S, Ramirez LC, Zaghini I, Chevillard G, Martin P, Pineau T & Bournot P 2004 Effects of peroxisome proliferator-activated receptor alpha activation on pathways contributing to cholesterol homeostasis in rat hepatocytes. *Biochim Biophys Acta* **1683** 49-58.

Lee BJ, Kang JK, Jung EY, Yun YW, Baek IJ, Yon JM, Lee YB, Sohn HS, Lee JY, Kim KS & Nam SY 2004a Exposure to genistein does not adversely affect the reproductive system in adult male mice adapted to a soy-based commercial diet. *J Vet Sci* **5** 227-234.

Lee BJ, Jung EY, Yun YW, Kang JK, Baek IJ, Yon JM, Lee YB, Sohn HS, Lee JY, Kim KS & Nam SY 2004b Effects of exposure to genistein during pubertal development on the reproductive system of male mice. *J Reprod Dev* **50** 399-409.

Leffers H, Naesby M, Vendelbo B, Skakkebaek NE & Jorgensen M 2001 Oestrogenic potencies of Zeranol, oestradiol, diethylstilboestrol, Bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. *Hum Reprod* **16** 1037-1045.

Leffler PE, Jin T & Nordberg GF 2000 Differential calcium transport disturbances in renal membrane vesicles after cadmium-metallothionein injection in rats. *Toxicology* **143** 227-234.

Lehman LD & Klaassen CD 1986 Dosage-dependent disposition of cadmium administered orally to rats. *Toxicol Appl Pharmacol* **84** 159-167.

Leopold AS, Erwin M, Oh J & Browning B 1976 Phytoestrogens: adverse effects on reproduction in California quail. *Science* **191** 98-100.

Leung AK & Robson WL 2007 Hypospadias: an update. *Asian J Androl* **9** 16-22.

Levy JR, Faber KA, Ayyash L & Hughes CL, Jr. 1995 The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc Soc Exp Biol Med* **208** 60-66.

Li ET & O'Dell BL 1986 Effect of zinc status on the binding of prostaglandins to ovarian membranes and intact platelets of pregnant rats. *J Nutr* **116** 1448-1455.

Lin H, Ge RS, Chen GR, Hu GX, Dong L, Lian QQ, Hardy DO, Sottas CM, Li XK & Hardy MP 2008 Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. *Proc Natl Acad Sci U S A* **105** 7218-7222.

Liu J, Liu Y, Habeebu SS & Klaassen CD 1998 Susceptibility of MT-null mice to chronic CdCl2-induced nephrotoxicity indicates that renal injury is not mediated by the CdMT complex. *Toxicol Sci* **46** 197-203.

Liu J, Corton C, Dix DJ, Liu Y, Waalkes MP & Klaassen CD 2001 Genetic background but not metallothionein phenotype dictates sensitivity to cadmium-induced testicular injury in mice. *Toxicol Appl Pharmacol* **176** 1-9.

Ljungvall K, Tienpont B, David F, Magnusson U & Torneke K 2004 Kinetics of orally administered di(2-ethylhexyl) phthalate and its metabolite, mono(2-ethylhexyl) phthalate, in male pigs. *Arch Toxicol* **78** 384-389.

Long GJ 1997 The effect of cadmium on cytosolic free calcium, protein kinase C, and collagen synthesis in rat osteosarcoma (ROS 17/2.8) cells. *Toxicol Appl Pharmacol* **143** 189-195.
Loomis AK & Thomas P 2000 Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor. *Biol Reprod* **62** 995-1004.
Loose DS, Kan PB, Hirst MA, Marcus RA & Feldman D 1983 Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. *J Clin Invest* **71** 1495-1499.
Lovekamp-Swan T & Davis BJ 2003 Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ Health Perspect* **111** 139-145.
Lu LJ & Anderson KE 1998 Sex and long-term soy diets affect the metabolism and excretion of soy isoflavones in humans. *Am J Clin Nutr* **68** 1500S-1504S.
Lundholm CD 1997 DDE-induced eggshell thinning in birds: effects of p,p'-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **118** 113-128.
Lymberopoulos AG, Kotsaki-Kovatsi VP, Taylor A, Papaioannou N & Brikas P 2000 Effects of cadmium chloride administration on the macroscopic and microscopic characteristics of ejaculates from Chios ram-lambs. *Theriogenology* **54** 1145-1157.
Ma M, Kondo T, Ban S, Umemura T, Kurahashi N, Takeda M & Kishi R 2006 Exposure of prepubertal female rats to inhaled di(2-ethylhexyl)phthalate affects the onset of puberty and postpubertal reproductive functions. *Toxicol Sci* **93** 164-171.
Madej A, Persson E, Lundh T & Ridderstrale Y 2002 Thyroid gland function in ovariectomized ewes exposed to phytoestrogens. *J Chromatogr B Analyt Technol Biomed Life Sci* **777** 281-287.
Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS & Sharpe RM 2005 Abnormal Leydig Cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* **146** 613-623.
Mahood IK, Scott HM, Brown R, Hallmark N, Walker M & Sharpe RM 2007 In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. *Environ Health Perspect* **115 Suppl 1** 55-61.
Manna PR, Tena-Sempere M & Huhtaniemi IT 1999 Molecular mechanisms of thyroid hormone-stimulated steroidogenesis in mouse leydig tumor cells. Involvement of the steroidogenic acute regulatory (StAR) protein. *J Biol Chem* **274** 5909-5918.
Manna PR, Wang XJ & Stocco DM 2003 Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids* **68** 1125-1134.
Manna PR, Kero J, Tena-Sempere M, Pakarinen P, Stocco DM & Huhtaniemi IT 2001 Assessment of mechanisms of thyroid hormone action in mouse Leydig cells: regulation of the steroidogenic acute regulatory protein, steroidogenesis, and luteinizing hormone receptor function. *Endocrinology* **142** 319-331.
Maran RR, Arunakaran J & Aruldhas MM 2000 T3 directly stimulates basal and modulates LH induced testosterone and oestradiol production by rat Leydig cells in vitro. *Endocr J* **47** 417-428.

Marty MS, Crissman JW & Carney EW 1999 Evaluation of the EDSTAC female pubertal assay in CD rats using 17beta-estradiol, steroid biosynthesis inhibitors, and a thyroid inhibitor. *Toxicol Sci* **52** 269-277.

Mather JP, Saez JM, Dray F & Haour F 1983 Vitamin E prolongs survival and function of porcine Leydig cells in culture. *Acta Endocrinol (Copenh)* **102** 470-475.

Mathieu AP, Auchus RJ & LeHoux JG 2002 Comparison of the hamster and human adrenal P450c17 (17 alpha-hydroxylase/17,20-lyase) using site-directed mutagenesis and molecular modeling. *J Steroid Biochem Mol Biol* **80** 99-107.

Mauduit C, Hartmann DJ, Chauvin MA, Revol A, Morera AM & Benahmed M 1991 Tumor necrosis factor alpha inhibits gonadotropin action in cultured porcine Leydig cells: site(s) of action. *Endocrinology* **129** 2933-2940.

Mauduit C, Gasnier F, Rey C, Chauvin MA, Stocco DM, Louisot P & Benahmed M 1998 Tumor necrosis factor-alpha inhibits leydig cell steroidogenesis through a decrease in steroidogenic acute regulatory protein expression. *Endocrinology* **139** 2863-2868.

McKee RH, El-Hawari M, Stoltz M, Pallas F & Lington AW 2002 Absorption, disposition and metabolism of di-isononyl phthalate (DINP) in F-344 rats. *J Appl Toxicol* **22** 293-302.

McKelvey W, Gwynn RC, Jeffery N, Kass D, Thorpe LE, Garg RK, Palmer CD & Parsons PJ 2007 A biomonitoring study of lead, cadmium, and mercury in the blood of New York city adults. *Environ Health Perspect* **115** 1435-1441.

McKinnell C, Sharpe RM, Mahood K, Hallmark N, Scott H, Ivell R, Staub C, Jegou B, Haag F, Koch-Nolte F & Hartung S 2005 Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by in utero exposure to di (n-Butyl) phthalate. *Endocrinology* **146** 4536-4544.

McNulty SE & Toscano WA, Jr. 1995 Transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochem Biophys Res Commun* **212** 165-171.

Meek ME & Chan PKL 1994 Bis(2-ethylhexyl)phthalate: evaluation of risks to health from environmental exposure in Canada. *J. Environ. Sci. Health. Part C.* **12** 179-194.

Meeker JD, Calafat AM & Hauser R 2007 Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. *Environ Health Perspect* **115** 1029-1034.

Menden EE, Elia VJ & Michael LW 1972 Distribution of cadmium and nickel of tobacco during cigarette smoking. *Environ. Sci. Technol.* **6** 830-832.

Menke A, Guallar E, Shiels MS, Rohrmann S, Basaria S, Rifai N, Nelson WG & Platz EA 2008 The association of urinary cadmium with sex steroid hormone concentrations in a general population sample of US adult men. *BMC Public Health* **8** 72.

Mgbonyebi OP, Smothers CT & Mrotek JJ 1994 Modulation of adrenal cell functions by cadmium salts: 3. Sites affected by CdCl2 during stimulated steroid synthesis. *Cell Biol Toxicol* **10** 35-43.

Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM & O'Brien DA 2004 Glyceraldehyde 3-phosphate dehydrogenase-S, a

sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc Natl Acad Sci U S A* **101** 16501-16506.
Miksicek RJ 1993 Commonly occurring plant flavonoids have estrogenic activity. *Mol Pharmacol* **44** 37-43.
Miksicek RJ 1994 Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *J Steroid Biochem Mol Biol* **49** 153-160.
Mitchell JH, Cawood E, Kinniburgh D, Provan A, Collins AR & Irvine DS 2001 Effect of a phytoestrogen food supplement on reproductive health in normal males. *Clin Sci (Lond)* **100** 613-618.
Molina-Molina JM, Hillenweck A, Jouanin I, Zalko D, Cravedi JP, Fernandez MF, Pillon A, Nicolas JC, Olea N & Balaguer P 2006 Steroid receptor profiling of vinclozolin and its primary metabolites. *Toxicol Appl Pharmacol* **216** 44-54.
Moller H 1998 Trends in sex-ratio, testicular cancer and male reproductive hazards: are they connected? *Apmis* **106** 232-238; discussion 238-239.
Montani C, Penza M, Jeremic M, Biasiotto G, La Sala G, De Felici M, Ciana P, Maggi A & Di Lorenzo D 2008 Genistein is an efficient estrogen in the whole-body throughout mouse development. *Toxicol Sci* **103** 57-67.
Moore W, Jr., Stara JF, Crocker WC, Malanchuk M & Iltis R 1973 Comparison of 115m cadmium retention in rats following different routes of administration. *Environ Res* **6** 473-478.
Mori T, Sumiya S & Yokota H 2000 Electrostatic interactions of androgens and progesterone derivatives with rainbow trout estrogen receptor. *J Steroid Biochem Mol Biol* **75** 129-137.
Morton MS, Arisaka O, Miyake N, Morgan LD & Evans BA 2002 Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. *J Nutr* **132** 3168-3171.
Mose T, Mortensen GK, Hedegaard M & Knudsen LE 2007a Phthalate monoesters in perfusate from a dual placenta perfusion system, the placenta tissue and umbilical cord blood. *Reprod Toxicol* **23** 83-91.
Mose T, Knudsen LE, Hedegaard M & Mortensen GK 2007b Transplacental transfer of monomethyl phthalate and mono(2-ethylhexyl) phthalate in a human placenta perfusion system. *Int J Toxicol* **26** 221-229.
Mueller PW, Paschal DC, Hammel RR, Klincewicz SL, MacNeil ML, Spierto B & Steinberg KK 1992 Chronic renal effects in three studies of men and women occupationally exposed to cadmium. *Arch Environ Contam Toxicol* **23** 125-136.
Mueller SO, Simon S, Chae K, Metzler M & Korach KS 2004 Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. *Toxicol Sci* **80** 14-25.
Mylchreest E, Cattley RC & Foster PM 1998 Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* **43** 47-60.
Mylchreest E, Sar M, Cattley RC & Foster PM 1999 Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* **156** 81-95.

Mylchreest E, Wallace DG, Cattley RC & Foster PM 2000 Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation. *Toxicol Sci* **55** 143-151.
Mylchreest E, Sar M, Wallace DG & Foster PM 2002 Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Reprod Toxicol* **16** 19-28.
Nasreddine L & Parent-Massin D 2002 Food contamination by metals and pesticides in the European Union. Should we worry? *Toxicol Lett* **127** 29-41.
Newbold RR 2004 Lessons learned from perinatal exposure to diethylstilbestrol. *Toxicol Appl Pharmacol* **199** 142-150.
Newbold RR & Jeffersson W 2005 Developmental and Reproductive Abnormalities Associated with Environmental Estrogens: Diethylstilbestrol (DES) as an Example. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, edn Second. Ed. RK Naz. Boca Raton: CRC Press.
Nilsson U, Schutz A, Skerfving S & Mattsson S 1995 Cadmium in kidneys in Swedes measured in vivo using X-ray fluorescence analysis. *Int Arch Occup Environ Health* **67** 405-411.
Nishijo M, Tawara K, Honda R, Nakagawa H, Tanebe K & Saito S 2004 Relationship between newborn size and mother's blood cadmium levels, Toyama, Japan. *Arch Environ Health* **59** 22-25.
Nishijo M, Nakagawa H, Honda R, Tanebe K, Saito S, Teranishi H & Tawara K 2002 Effects of maternal exposure to cadmium on pregnancy outcome and breast milk. *Occup Environ Med* **59** 394-396; discussion 397.
Niswender GD & Nett TM 1994 Corpus luteum and its control in infraprimate species. In *The physiology of reproduction, vol 1* Eds E Knobil & JD Neill. New York: Raven.
Niswender GD, Juengel JL, Silva PJ, Rollyson MK & McIntush EW 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* **80** 1-29.
Nordberg G 1971 Effects of Acute and Chronic Cadmium Exposure on the Testicles of Mice. *Env. Physiol.* **1** 171-187.
Nordberg G, Jin T, Bernard A, Fierens S, Buchet JP, Ye T, Kong Q & Wang H 2002 Low bone density and renal dysfunction following environmental cadmium exposure in China. *Ambio* **31** 478-481.
Nordberg GF 1972 Cadmium metabolism and toxicity. Experimental studies on mice with special reference to the use of biological materials as indicies of retention and the possible role of metallothionein in transport and detoxification of cadmium. *Environ. Physiol. Biochem.* **2** 7-36.
Nordberg GF & Nordberg M 1987 Different binding forms of cadmium--implications for distribution and toxicity. *J Uoeh* **9 Suppl** 153-164.
Nordberg GF, Kjellstrom T & Nordberg M 1985 Kinetics and metabolism. In *Cadmium and Health: A Toxicological and Epidemiological Appraisal. Vol.1. Exposure, Dose and Metabolism*. Eds L Friberg, CG Elinder, T Kjellstrom & GF Nordberg. Boca Raton: CRC Press.

Nordberg GF, Nogawa K, Nordberg M & Friberg LT 2007 Cadmium (Chapter 23). In *Handbook on the Toxicology of Metals*, edn Third. Eds GF Nordberg, L Friberg & VB Vouk. Burlington: Academic Press.
Nordberg M & Nordberg GF 1975 Distribution of metallothionein-bound cadmium and cadmium chloride in mice: preliminary studies. *Environ Health Perspect* **12** 103-108.
Nordberg M, Winblad B & Basun H 2000 Cadmium concentration in blood in an elderly urban population. *Biometals* **13** 311-317.
Okey AB, Franc MA, Moffat ID, Tijet N, Boutros PC, Korkalainen M, Tuomisto J & Pohjanvirta R 2005 Toxicological implications of polymorphisms in receptors for xenobiotic chemicals: The case of the aryl hydrocarbon receptor. *Toxicol Appl Pharmacol* **207** 43-51.
Olofsson J, Norjavaara E & Selstam G 1990 In vivo levels of prostaglandin F2 alpha, E2 and prostacyclin in the corpus luteum of pregnant and pseudopregnant rats. *Biol Reprod* **42** 792-800.
Opalka DM, Kaminska B, Piskula MK, Puchajda-Skowronska H & Dusza L 2006 Effects of phytoestrogens on testosterone secretion by Leydig cells from Bilgoraj ganders (Anser anser). *Br Poult Sci* **47** 237-245.
Opalka M, Kaminska B, Ciereszko R & Dusza L 2004 Genistein affects testosterone secretion by Leydig cells in roosters (Gallus gallus domesticus). *Reprod Biol* **4** 185-193.
Orlicky DJ & Williams-Skipp C 1992 Immunohistochemical localization of PGF2 alpha receptor in the mouse testis. *Prostaglandins Leukot Essent Fatty Acids* **47** 247-252.
Ouellette AJ, Aviles L, Burnweit CA, Frederick D & Malt RA 1982 Metallothionein mRNA induction in mouse small bowel by oral cadmium and zinc. *Am J Physiol* **243** G396-403.
Ozawa N, Goda N, Makino N, Yamaguchi T, Yoshimura Y & Suematsu M 2002 Leydig cell-derived heme oxygenase-1 regulates apoptosis of premeiotic germ cells in response to stress. *J Clin Invest* **109** 457-467.
Pan L, Xia X, Feng Y, Jiang C, Cui Y & Huang Y 2008 Exposure of juvenile rats to the phytoestrogen daidzein impairs erectile function in a dose-related manner in adulthood. *J Androl* **29** 55-62.
Papadopoulos V, Amri H, Li H, Boujrad N, Vidic B & Garnier M 1997 Targeted disruption of the peripheral-type benzodiazepine receptor gene inhibits steroidogenesis in the R2C Leydig tumor cell line. *J Biol Chem* **272** 32129-32135.
Parizek J & Zahor Z 1956 Effect of cadmium salts on testicular tissue. *Nature* **177** 1036.
Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ & Gray LE, Jr. 2000 The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci* **58** 339-349.
Patisaul HB & Whitten PL 2005 Dietary Phytoestrogens. In *Endocrine Disruptors: Effects on Male and Female Reproductive Systems*, edn Second. Ed. RK Naz. Boca Raton: CRC Press.

Paulozzi LJ, Erickson JD & Jackson RJ 1997 Hypospadias trends in two US surveillance systems. *Pediatrics* **100** 831-834.
Paulsen CA, Berman NG & Wang C 1996 Data from men in greater Seattle area reveals no downward trend in semen quality: further evidence that deterioration of semen quality is not geographically uniform. *Fertil Steril* **65** 1015-1020.
Payne AH & Youngblood GL 1995 Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol Reprod* **52** 217-225.
Pelletier G & El-Alfy M 2000 Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. *J Clin Endocrinol Metab* **85** 4835-4840.
Piasek M & Laskey JW 1994 Acute cadmium exposure and ovarian steroidogenesis in cycling and pregnant rats. *Reprod Toxicol* **8** 495-507.
Piasek M & Laskey JW 1999 Effects of in vitro cadmium exposure on ovarian steroidogenesis in rats. *J Appl Toxicol* **19** 211-217.
Piasek M, Blanusa M, Kostial K & Laskey JW 2001 Placental cadmium and progesterone concentrations in cigarette smokers. *Reprod Toxicol* **15** 673-681.
Piasek M, Laskey JW, Kostial K & Blanusa M 2002 Assessment of steroid disruption using cultures of whole ovary and/or placenta in rat and in human placental tissue. *Int Arch Occup Environ Health* **75 Suppl** S36-44.
Poliandri AH, Cabilla JP, Velardez MO, Bodo CC & Duvilanski BH 2003 Cadmium induces apoptosis in anterior pituitary cells that can be reversed by treatment with antioxidants. *Toxicol Appl Pharmacol* **190** 17-24.
Porter RD & Wiemeyer SN 1969 Dieldrin and DDT: effects on sparrow hawk eggshells and reproduction. *Science* **165** 199-200.
Potashnik G, Yanai-Inbar I, Sacks MI & Israeli R 1979 Effect of dibromochloropropane on human testicular function. *Isr J Med Sci* **15** 438-442.
Potashnik G, Ben-Aderet N, Israeli R, Yanai-Inbar I & Sober I 1978 Suppressive effect of 1,2-dibromo-3-chloropropane on human spermatogenesis. *Fertil Steril* **30** 444-447.
Powell SR 2000 The antioxidant properties of zinc. *J Nutr* **130** 1447S-1454S.
Priya PN, Pillai A & Gupta S 2004 Effect of simultaneous exposure to lead and cadmium on gonadotropin binding and steroidogenesis on granulosa cells: an in vitro study. *Indian J Exp Biol* **42** 143-148.
Ramirez DC & Gimenez MS 2003 Induction of redox changes, inducible nitric oxide synthase and cyclooxygenase-2 by chronic cadmium exposure in mouse peritoneal macrophages. *Toxicol Lett* **145** 121-132.
Ratcliffe DA 1967 Decrease in eggshell weight in certain birds of prey. *Nature* **215** 208-210.
Reddy BS, Rozati R, Reddy BV & Raman NV 2006 Association of phthalate esters with endometriosis in Indian women. *Bjog* **113** 515-520.
Ren XY, Zhou Y, Zhang JP, Feng WH & Jiao BH 2003a Metallothionein gene expression under different time in testicular Sertoli and spermatogenic cells of rats treated with cadmium. *Reprod Toxicol* **17** 219-227.

Ren XY, Zhou Y, Zhang JP, Feng WH & Jiao BH 2003b Expression of metallothionein gene at different time in testicular interstitial cells and liver of rats treated with cadmium. *World J Gastroenterol* **9** 1554-1558.
Rhodes C, Orton TC, Pratt IS, Batten PL, Bratt H, Jackson SJ & Elcombe CR 1986 Comparative pharmacokinetics and subacute toxicity of di(2-ethylhexyl) phthalate (DEHP) in rats and marmosets: extrapolation of effects in rodents to man. *Environ Health Perspect* **65** 299-307.
Ricard AC, Daniel C, Anderson P & Hontela A 1998 Effects of subchronic exposure to cadmium chloride on endocrine and metabolic functions in rainbow trout Oncorhynchus mykiss. *Arch Environ Contam Toxicol* **34** 377-381.
Richiardi L, Bellocco R, Adami HO, Torrang A, Barlow L, Hakulinen T, Rahu M, Stengrevics A, Storm H, Tretli S, Kurtinaitis J, Tyczynski JE & Akre O 2004 Testicular cancer incidence in eight northern European countries: secular and recent trends. *Cancer Epidemiol Biomarkers Prev* **13** 2157-2166.
Rikans LE & Yamano T 2000 Mechanisms of cadmium-mediated acute hepatotoxicity. *J Biochem Mol Toxicol* **14** 110-117.
Ripple MO & Wilding G 1995 Alteration of glyceraldehyde-3-phosphate dehydrogenase activity and messenger RNA content by androgen in human prostate carcinoma cells. *Cancer Res* **55** 4234-4236.
Ryu DY, Lee SJ, Park DW, Choi BS, Klaassen CD & Park JD 2004 Dietary iron regulates intestinal cadmium absorption through iron transporters in rats. *Toxicol Lett* **152** 19-25.
Saaranen M, Kantola M, Saarikoski S & Vanha-Perttula T 1989 Human seminal plasma cadmium: comparison with fertility and smoking habits. *Andrologia* **21** 140-145.
Safe SH 1995 Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *Pharmacol Ther* **67** 247-281.
Saksena SK, el-Safoury S & Bartke A 1973 Prostaglandins E2 and F2 decrease plasma testosterone levels in male rats. *Prostaglandins* **4** 235-242.
Saldivar L, Luna M, Reyes E, Soto R & Fortoul TI 1991 Cadmium determination in Mexican-produced tobacco. *Environ Res* **55** 91-96.
Salpietro CD, Gangemi S, Minciullo PL, Briuglia S, Merlino MV, Stelitano A, Cristani M, Trombetta D & Saija A 2002 Cadmium concentration in maternal and cord blood and infant birth weight: a study on healthy non-smoking women. *J Perinat Med* **30** 395-399.
Satarug S & Moore MR 2004 Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. *Environ Health Perspect* **112** 1099-1103.
Satarug S, Baker JR, Reilly PE, Moore MR & Williams DJ 2002 Cadmium levels in the lung, liver, kidney cortex, and urine samples from Australians without occupational exposure to metals. *Arch Environ Health* **57** 69-77.
Satarug S, Baker JR, Urbenjapol S, Haswell-Elkins M, Reilly PE, Williams DJ & Moore MR 2003 A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol Lett* **137** 65-83.

Saunders PT, Sharpe RM, Williams K, Macpherson S, Urquart H, Irvine DS & Millar MR 2001 Differential expression of oestrogen receptor alpha and beta proteins in the testes and male reproductive system of human and non-human primates. *Mol Hum Reprod* **7** 227-236.
Savage MO & Lowe DG 1990 Gonadal neoplasia and abnormal sexual differentiation. *Clin Endocrinol (Oxf)* **32** 519-533.
Schmitt E, Dekant W & Stopper H 2001 Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. *Toxicol In Vitro* **15** 433-439.
Scott HM, Hutchison GR, Jobling MS, McKinnell C, Drake AJ & Sharpe RM 2008 Relationship between androgen action in the 'male programming window', fetal sertoli cell number and adult testis size in the rat. *Endocrinology*.
Sen Gupta R, Sen Gupta E, Dhakal BK, Thakur AR & Ahnn J 2004a Vitamin C and vitamin E protect the rat testes from cadmium-induced reactive oxygen species. *Mol Cells* **17** 132-139.
Sen Gupta R, Kim J, Gomes C, Oh S, Park J, Im WB, Seong JY, Ahn RS, Kwon HB & Soh J 2004b Effect of ascorbic acid supplementation on testicular steroidogenesis and germ cell death in cadmium-treated male rats. *Mol Cell Endocrinol* **221** 57-66.
Setchell KD, Brown NM, Zimmer-Nechemias L, Brashear WT, Wolfe BE, Kirschner AS & Heubi JE 2002 Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr* **76** 447-453.
Setchell KD, Gosselin SJ, Welsh MB, Johnston JO, Balistreri WF, Kramer LW, Dresser BL & Tarr MJ 1987 Dietary estrogens--a probable cause of infertility and liver disease in captive cheetahs. *Gastroenterology* **93** 225-233.
Shaham J, Meltzer A, Ashkenazi R & Ribak J 1996 Biological monitoring of exposure to cadmium, a human carcinogen, as a result of active and passive smoking. *J Occup Environ Med* **38** 1220-1228.
Sharief FS, Mohler JL, Sharief Y & Li SS 1994 Expression of human prostatic acid phosphatase and prostate specific antigen genes in neoplastic and benign tissues. *Biochem Mol Biol Int* **33** 567-574.
Sharpe RM & Skakkebaek NE 2008 Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. *Fertil Steril* **89** e33-38.
Shea KM 2003 Pediatric exposure and potential toxicity of phthalate plasticizers. *Pediatrics* **111** 1467-1474.
Shono T, Suita S, Kai H & Yamaguchi Y 2004 The effect of a prenatal androgen disruptor, vinclozolin, on gubernacular migration and testicular descent in rats. *J Pediatr Surg* **39** 213-216.
Shutt DA & Cox RI 1972 Steroid and phyto-oestrogen binding to sheep uterine receptors in vitro. *J Endocrinol* **52** 299-310.
Silva MJ, Samandar E, Reidy JA, Hauser R, Needham LL & Calafat AM 2007 Metabolite profiles of di-n-butyl phthalate in humans and rats. *Environ Sci Technol* **41** 7576-7580.

Silva MJ, Barr DB, Reidy JA, Kato K, Malek NA, Hodge CC, Hurtz D, 3rd, Calafat AM, Needham LL & Brock JW 2003 Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. *Arch Toxicol* **77** 561-567.
Simanainen U, Adamsson A, Tuomisto JT, Miettinen HM, Toppari J, Tuomisto J & Viluksela M 2004a Adult 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure and effects on male reproductive organs in three differentially TCDD-susceptible rat lines. *Toxicol Sci* **81** 401-407.
Simanainen U, Haavisto T, Tuomisto JT, Paranko J, Toppari J, Tuomisto J, Peterson RE & Viluksela M 2004b Pattern of male reproductive system effects after in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure in three differentially TCDD-sensitive rat lines. *Toxicol Sci* **80** 101-108.
Sirover MA 1997 Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and in cell pathology. *J Cell Biochem* **66** 133-140.
Sirover MA 1999 New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* **1432** 159-184.
Sjoberg P, Bondesson U, Gray TJ & Ploen L 1986 Effects of di-(2-ethylhexyl) phthalate and five of its metabolites on rat testis in vivo and in in vitro. *Acta Pharmacol Toxicol (Copenh)* **58** 225-233.
Sjoberg PO, Bondesson UG, Sedin EG & Gustafsson JP 1985 Exposure of newborn infants to plasticizers. Plasma levels of di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate during exchange transfusion. *Transfusion* **25** 424-428.
Skakkebaek NE, Rajpert-De Meyts E & Main KM 2001 Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* **16** 972-978.
Smida AD, Valderrama XP, Agostini MC, Furlan MA & Chedrese J 2004 Cadmium stimulates transcription of the cytochrome p450 side chain cleavage gene in genetically modified stable porcine granulosa cells. *Biol Reprod* **70** 25-31.
Smith OW & Smith GV 1949a The influence of diethylstilbestrol on the progress and outcome of pregnancy as based on a comparison of treated with untreated primigravidas. *Am J Obstet Gynecol* **58** 994-1009.
Smith OW & Smith GV 1949b Use of diethylstilbestrol to prevent fetal loss from complications of late pregnancy. *N Engl J Med* **241** 562-568.
Song KB, Atkinson C, Frankenfeld CL, Jokela T, Wahala K, Thomas WK & Lampe JW 2006 Prevalence of daidzein-metabolizing phenotypes differs between Caucasian and Korean American women and girls. *J Nutr* **136** 1347-1351.
Souza V, Escobar Mdel C, Bucio L, Hernandez E & Gutierrez-Ruiz MC 2004 Zinc pretreatment prevents hepatic stellate cells from cadmium-produced oxidative damage. *Cell Biol Toxicol* **20** 241-251.
Steinshamn H, Purup S, Thuen E & Hansen-Moller J 2008 Effects of clover-grass silages and concentrate supplementation on the content of phytoestrogens in dairy cow milk. *J Dairy Sci* **91** 2715-2725.
Stohs SJ, Bagchi D, Hassoun E & Bagchi M 2000 Oxidative mechanisms in the toxicity of chromium and cadmium ions. *J Environ Pathol Toxicol Oncol* **19** 201-213.

Stoica A, Katzenellenbogen BS & Martin MB 2000 Activation of estrogen receptor-alpha by the heavy metal cadmium. *Mol Endocrinol* **14** 545-553.
Stroheker T, Picard K, Lhuguenot JC, Canivenc-Lavier MC & Chagnon MC 2004 Steroid activities comparison of natural and food wrap compounds in human breast cancer cell lines. *Food Chem Toxicol* **42** 887-897.
Stroheker T, Cabaton N, Nourdin G, Regnier JF, Lhuguenot JC & Chagnon MC 2005 Evaluation of anti-androgenic activity of di-(2-ethylhexyl)phthalate. *Toxicology* **208** 115-121.
Strom BL, Schinnar R, Ziegler EE, Barnhart KT, Sammel MD, Macones GA, Stallings VA, Drulis JM, Nelson SE & Hanson SA 2001 Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood. *Jama* **286** 807-814.
Sunderman FW, Jr., Plowman MC & Hopfer SM 1992 Teratogenicity of cadmium chloride in the South African frog, Xenopus laevis. *IARC Sci Publ* 249-256.
Suzuki-Yamamoto T, Sugimoto Y, Ichikawa A & Ishimura K 2007 Co-localization of prostaglandin F synthase, cyclooxygenase-1 and prostaglandin F receptor in mouse Leydig cells. *Histochem Cell Biol* **128** 317-322.
Suzuki T, Momoi K, Hosoyamada M, Kimura M & Shibasaki T 2008 Normal cadmium uptake in microcytic anemia mk/mk mice suggests that DMT1 is not the only cadmium transporter in vivo. *Toxicol Appl Pharmacol* **227** 462-467.
Svechnikov K, Supornsilchai V, Strand ML, Wahlgren A, Seidlova-Wuttke D, Wuttke W & Soder O 2005 Influence of long-term dietary administration of procymidone, a fungicide with anti-androgenic effects, or the phytoestrogen genistein to rats on the pituitary-gonadal axis and Leydig cell steroidogenesis. *J Endocrinol* **187** 117-124.
Svechnikova I, Svechnikov K & Soder O 2007 The influence of di-(2-ethylhexyl) phthalate on steroidogenesis by the ovarian granulosa cells of immature female rats. *J Endocrinol* **194** 603-609.
Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S & Teague JL 2005 Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* **113** 1056-1061.
Szczerbik P, Mikolajczyk T, Sokolowska-Mikolajczyk M, Socha M, Chyb J & Epler P 2006 Influence of long-term exposure to dietary cadmium on growth, maturation and reproduction of goldfish (subspecies: Prussian carp Carassius auratus gibelio B.). *Aquat Toxicol* **77** 126-135.
Tabb MM & Blumberg B 2006 New modes of action for endocrine-disrupting chemicals. *Mol Endocrinol* **20** 475-482.
Takeuchi S, Iida M, Kobayashi S, Jin K, Matsuda T & Kojima H 2005 Differential effects of phthalate esters on transcriptional activities via human estrogen receptors alpha and beta, and androgen receptor. *Toxicology* **210** 223-233.
Tanaka A, Matsumoto A & Yamaha T 1978 Biochemical studies on phthalic esters. III. Metabolism of dibutyl phthalate (DBP) in animals. *Toxicology* **9** 109-123.

Teas J, Braverman LE, Kurzer MS, Pino S, Hurley TG & Hebert JR 2007 Seaweed and soy: companion foods in Asian cuisine and their effects on thyroid function in American women. *J Med Food* **10** 90-100.
Thomas P 2008 Characteristics of membrane progestin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Front Neuroendocrinol* **29** 292-312.
Thomas P & Dong J 2006 Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J Steroid Biochem Mol Biol* **102** 175-179.
Thomas P, Dressing G, Pang Y, Berg H, Tubbs C, Benninghoff A & Doughty K 2006 Progestin, estrogen and androgen G-protein coupled receptors in fish gonads. *Steroids* **71** 310-316.
Thompson J & Bannigan J 2008 Cadmium: toxic effects on the reproductive system and the embryo. *Reprod Toxicol* **25** 304-315.
Thompson LU, Robb P, Serraino M & Cheung F 1991 Mammalian lignan production from various foods. *Nutr Cancer* **16** 43-52.
Toledano MB, Jarup L, Best N, Wakefield J & Elliott P 2001 Spatial variation and temporal trends of testicular cancer in Great Britain. *Br J Cancer* **84** 1482-1487.
Tomar RS & Shiao R 2008 Early life and adult exposure to isoflavones and breast cancer risk. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **26** 113-173.
Trentacoste SV, Friedmann AS, Youker RT, Breckenridge CB & Zirkin BR 2001 Atrazine effects on testosterone levels and androgen-dependent reproductive organs in peripubertal male rats. *J Androl* **22** 142-148.
Tsoumbaris P & Tsoukali-Papadopoulou H 1994 Heavy metals in common foodstuff: daily intake. *Bull Environ Contam Toxicol* **53** 67-70.
Tyl RW, Myers CB, Marr MC, Fail PA, Seely JC, Brine DR, Barter RA & Butala JH 2004 Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. *Reprod Toxicol* **18** 241-264.
Uehar M, Arai Y, Watanabe S & Adlercreutz H 2000 Comparison of plasma and urinary phytoestrogens in Japanese and Finnish women by time-resolved fluoroimmunoassay. *Biofactors* **12** 217-225.
Vajda AM & Norris DO 2006 Endocrine-Active Phytochemicals: Environmental Signaling Context and Mechanisms. In *Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans*. Eds DO Norris & JA Carr. New York: Oxford University Press.
Valentin-Blasini L, Sadowski MA, Walden D, Caltabiano L, Needham LL & Barr DB 2005 Urinary phytoestrogen concentrations in the U.S. population (1999-2000). *J Expo Anal Environ Epidemiol* **15** 509-523.
Varga B, Zsolnai B, Paksy K, Naray M & Ungvary G 1993 Age dependent accumulation of cadmium in the human ovary. *Reprod Toxicol* **7** 225-228.
Venkata NG, Robinson JA, Cabot PJ, Davis B, Monteith GR & Roberts-Thomson SJ 2006 Mono(2-ethylhexyl)phthalate and mono-n-butyl phthalate activation of peroxisome proliferator activated-receptors alpha and gamma in breast. *Toxicol Lett* **163** 224-234.

Virtanen HE, Rajpert-De Meyts E, Main KM, Skakkebaek NE & Toppari J 2005 Testicular dysgenesis syndrome and the development and occurrence of male reproductive disorders. *Toxicol Appl Pharmacol* **207** 501-505.
Virtanen HE, Cortes D, Rajpert-De Meyts E, Ritzen EM, Nordenskjold A, Skakkebaek NE & Toppari J 2007 Development and descent of the testis in relation to cryptorchidism. *Acta Paediatr* **96** 622-627.
Virtanen HE, Kaleva M, Haavisto AM, Schmidt IM, Chellakooty M, Main KM, Skakkebaek NE & Toppari J 2001 The birth rate of hypospadias in the Turku area in Finland. *Apmis* **109** 96-100.
Waalkes MP & Perantoni A 1988 In vitro assessment of target cell specificity in cadmium carcinogenesis: interactions of cadmium and zinc with isolated interstitial cells of the rat testes. *In Vitro Cell Dev Biol* **24** 558-565.
Waalkes MP, Chernoff SB & Klaassen CD 1984 Cadmium-binding proteins of rat testes. Apparent source of the protein of low molecular mass. *Biochem J* **220** 819-824.
Waalkes MP, Rehm S, Perantoni AO & Coogan TP 1992 Cadmium exposure in rats and tumours of the prostate. *IARC Sci Publ* 391-400.
Waalkes MP, Rehm S, Riggs CW, Bare RM, Devor DE, Poirier LA, Wenk ML, Henneman JR & Balaschak MS 1988 Cadmium carcinogenesis in male Wistar [Crl:(WI)BR] rats: dose-response analysis of tumor induction in the prostate and testes and at the injection site. *Cancer Res* **48** 4656-4663.
Walschaerts M, Huyghe E, Muller A, Bachaud JM, Bujan L & Thonneau P 2008 Doubling of testicular cancer incidence rate over the last 20 years in southern France. *Cancer Causes Control* **19** 155-161.
Walsh DE, Dockery P & Doolan CM 2005 Estrogen receptor independent rapid non-genomic effects of environmental estrogens on [Ca2+]i in human breast cancer cells. *Mol Cell Endocrinol* **230** 23-30.
Wang LQ 2002 Mammalian phytoestrogens: enterodiol and enterolactone. *J Chromatogr B Analyt Technol Biomed Life Sci* **777** 289-309.
Watanabe S, Terashima K, Sato Y, Arai S & Eboshida A 2000 Effects of isoflavone supplement on healthy women. *Biofactors* **12** 233-241.
Watanabe T, Shimbo S, Nakatsuka H, Koizumi A, Higashikawa K, Matsuda-Inoguchi N & Ikeda M 2004 Gender-related difference, geographical variation and time trend in dietary cadmium intake in Japan. *Sci Total Environ* **329** 17-27.
Waters KM, Safe S & Gaido KW 2001 Differential gene expression in response to methoxychlor and estradiol through ERalpha, ERbeta, and AR in reproductive tissues of female mice. *Toxicol Sci* **63** 47-56.
Webb M 1972 Protection by zinc against cadmium toxicity. *Biochem Pharmacol* **21** 2767-2771.
Webster WS & Messerle K 1980 Changes in the mouse neuroepithelium associated with cadmium-induced neural tube defects. *Teratology* **21** 79-88.
Weiss J, Axelrod L, Whitcomb RW, Harris PE, Crowley WF & Jameson JL 1992 Hypogonadism caused by a single amino acid substitution in the beta subunit of luteinizing hormone. *N Engl J Med* **326** 179-183.
Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB & Sharpe RM 2008 Identification in rats of a programming window for reproductive tract

masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* **118** 1479-1490.
WHO 1989 Evaluation of Certain Food Additives and Contaminants (Thirty-third Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 776. Geneva: World Health Organization.
WHO 1992 Cadmium. Environmental Health Criteria. Vol. 134. Geneva: World Health Organization.
WHO 2004 Evaluation of Certain Food Additives and Contaminants. WHO Technical Report Series. No.922, 1-176. Geneva: World Health Organization.
Willers S, Attewell R, Bensryd I, Schutz A, Skarping G & Vahter M 1992 Exposure to environmental tobacco smoke in the household and urinary cotinine excretion, heavy metals retention, and lung function. *Arch Environ Health* **47** 357-363.
Wilson VS, Bobseine K & Gray LE, Jr. 2004a Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci* **81** 69-77.
Wilson VS, Howdeshell KL, Lambright CS, Furr J & Earl Gray L, Jr. 2007 Differential expression of the phthalate syndrome in male Sprague-Dawley and Wistar rats after in utero DEHP exposure. *Toxicol Lett* **170** 177-184.
Wilson VS, Lambright C, Furr J, Ostby J, Wood C, Held G & Gray LE, Jr. 2004b Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol Lett* **146** 207-215.
Winstel C & Callahan P 1992 Cadmium exposure inhibits the prolactin secretory response to thyrotrophin releasing hormone (TRH) in vitro. *Toxicology* **74** 9-17.
Wisniewski AB, Klein SL, Lakshmanan Y & Gearhart JP 2003 Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. *J Urol* **169** 1582-1586.
Wittassek M & Angerer J 2008 Phthalates: metabolism and exposure. *Int J Androl* **31** 131-138.
Wittassek M, Wiesmuller GA, Koch HM, Eckard R, Dobler L, Muller J, Angerer J & Schluter C 2007 Internal phthalate exposure over the last two decades--a retrospective human biomonitoring study. *Int J Hyg Environ Health* **210** 319-333.
Wolff MS, Britton JA, Boguski L, Hochman S, Maloney N, Serra N, Liu Z, Berkowitz G, Larson S & Forman J 2008 Environmental exposures and puberty in inner-city girls. *Environ Res* **107** 393-400.
Wong C, Kelce WR, Sar M & Wilson EM 1995 Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J Biol Chem* **270** 19998-20003.
Yang JM, Arnush M, Chen QY, Wu XD, Pang B & Jiang XZ 2003 Cadmium-induced damage to primary cultures of rat Leydig cells. *Reprod Toxicol* **17** 553-560.
Yang P, Kriatchko A & Roy SK 2002 Expression of ER-alpha and ER-beta in the hamster ovary: differential regulation by gonadotropins and ovarian steroid hormones. *Endocrinology* **143** 2385-2398.
Yong EL, Loy CJ & Sim KS 2003 Androgen receptor gene and male infertility. *Hum Reprod Update* **9** 1-7.

Zalups RK & Ahmad S 2003 Molecular handling of cadmium in transporting epithelia. *Toxicol Appl Pharmacol* **186** 163-188.
Zeng X, Jin T, Zhou Y & Nordberg GF 2003 Changes of serum sex hormone levels and MT mRNA expression in rats orally exposed to cadmium. *Toxicology* **186** 109-118.
Zeng X, Jin T, Buchet JP, Jiang X, Kong Q, Ye T, Bernard A & Nordberg GF 2004 Impact of cadmium exposure on male sex hormones: a population-based study in China. *Environ Res* **96** 338-344.
Zhang FP, Poutanen M, Wilbertz J & Huhtaniemi I 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol* **15** 172-183.
Zhang W & Jia H 2007 Effect and mechanism of cadmium on the progesterone synthesis of ovaries. *Toxicology* **239** 204-212.
Zhang ZW, Moon CS, Watanabe T, Shimbo S, He FS, Wu YQ, Zhou SF, Su DM, Qu JB & Ikeda M 1997 Background exposure of urban populations to lead and cadmium: comparison between China and Japan. *Int Arch Occup Environ Health* **69** 273-281.
Zhou D & Chen S 1999 Identification and characterization of a cAMP-responsive element in the region upstream from promoter 1.3 of the human aromatase gene. *Arch Biochem Biophys* **371** 179-190.
Zhou T, Zhou G, Song W, Eguchi N, Lu W, Lundin E, Jin T & Nordberg G 1999 Cadmium-induced apoptosis and changes in expression of p53, c-jun and MT-I genes in testes and ventral prostate of rats. *Toxicology* **142** 1-13.
Zhu BT, Han GZ, Shim JY, Wen Y & Jiang XR 2006a Quantitative structure-activity relationship of various endogenous estrogen metabolites for human estrogen receptor alpha and beta subtypes: Insights into the structural determinants favoring a differential subtype binding. *Endocrinology* **147** 4132-4150.
Zhu J, Phillips SP, Feng YL & Yang X 2006b Phthalate esters in human milk: concentration variations over a 6-month postpartum time. *Environ Sci Technol* **40** 5276-5281.
Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W & Adham IM 1999 Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol Endocrinol* **13** 681-691.
Zirkin BR & Chen H 2000 Regulation of Leydig cell steroidogenic function during aging. *Biol Reprod* **63** 977-981.

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