Prepubertal Exposure to Di(2-ethylhexyl) Phthalate

Kinetics and Effects on the Reproductive System of the Boar

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006: 15

ISSN 1652-6880
ISBN 91-576-7064-1

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Ljungvall, K. 2006. *Prepubertal Exposure to Di(2-ethylhexyl) Phthalate- Kinetics and Effects on the Reproductive System of the Boar. Doctor's dissertation.*
ISSN 1652-6880, ISBN 91-576-7064-1

The aims of this thesis were to increase the knowledge about endocrine disruption and the relations between prepubertal exposure and delayed, long-lasting effects on the reproductive system. Furthermore, the generality of knowledge in reproductive toxicology, generated in rodents was challenged by using a non-rodent species, the pig.

In two different experimental sets the immediate and late effects of prepubertal exposure to low repeated doses of the abundant plasticizer di(2-ethylhexyl) phthalate (DEHP) on several reproductive traits were investigated in boars. In an additional experiment, the kinetics of DEHP and mono(2-ethylhexyl) phthalate (MEHP the primary, bio-active metabolite of DEHP) in the boar were investigated.

After parenteral exposure to DEHP for five weeks, from the sixth week of age, the plasma concentrations of testosterone were higher and the area of the Leydig cells larger at 7.5 months, compared with the control group. Because the plasma concentrations of LH were unaffected, these data suggest that DEHP early in life causes long-lasting derangements in the fine tuning of the feedback loop in the hypothalamic-pituitary-gonadal axis (HPG-axis).

After oral exposure to DEHP for four weeks, from the fourth week of age, LH profiles of the exposed and non-exposed boars differed slightly, both during the exposure period, and after stimulation with a GnRH analogue at nine months of age. These results corroborate the hypothesis that DEHP is an endocrine disruptor following prepubertal exposure. In the same pigs orally exposed to DEHP, the gross morphology as well as the microscopic morphology of the testes was unaffected at seven weeks of age. However, at nine months of age, the bulbourethral glands were larger in the boars exposed to although the microscopic morphology of the testes was unaffected. In addition, the mating behaviour of the boars was examined between six and nine months of age. The libido as well as the mating success was tested to determine whether DEHP affected the central nervous system. However, the mating behaviour and mating ability of DEHP-exposed boars was found to be unaffected.

In young boars the concentrations of MEHP in plasma after oral exposure to DEHP were analyzed. It seemed that the systemic exposure to MEHP was lower in pigs compared to rats at the same oral dosage of DEHP. This may also explain some of the differences in the effects of this compound in different species.

While corroborating the hypothesis that prepubertal exposure to the industrial chemical DEHP affects the reproductive endocrinology in mammals, the contents of this work do not suggest any behavioural effects of DEHP in mammals. It is

noteworthy that the effects seen on testosterone concentrations and bulbourethral gland size are seen in the boars after the onset of puberty and not at the time of exposure. In view of the above, the use of pigs as a non-rodent complement in the field of reproductive toxicology is relevant.

Keywords: behaviour, boar, endocrine disruption, endocrinology, DEHP, testis

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. 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. *Archives of Toxicology 78*, 384-9. With kind permission from Springer Science and Business Media.

II. Ljungvall K, Karlsson P, Hulten F, Madej A, Norrgren L, Einarsson S, Rodriguez-Martinez H, Magnusson U. 2005. Delayed effects on plasma concentration of testosterone and testicular morphology by intramuscular low-dose di(2-ethylhexyl)phthalate or oestradiol benzoate in the prepubertal boar. *Theriogenology*. 64, 1170-84.With kind permission from Elsevier.

III. Ljungvall K, Spjuth L, Hulten F, Einarsson S, Rodriguez-Martinez H, Andersson K, Magnusson U. 2006. Early post-natal exposure to low dose oral di(2ethylhexyl) phthalate affects the peripheral LH-concentration in plasma, but does not affect mating behavior in the post-pubertal boar. *Reproductive Toxicology* 21, 160-6. With kind permission from Elsevier.

IV. Ljungvall K, Hultén F, Magnusson U. 2006. Morphology and morphometry of the reproductive organs in both prepubertal and postpubertal male pigs exposed to Di(2ethylhexyl) Phthalate before puberty. *Manuscript*.

Abbreviations

DEHP	Di(2-ethylhexyl) Phthalate
EDC	Endocrine Disrupting Chemical
GnRH	Gonadotrophin Releasing Hormone
HPG	Hypothalamic-Pituitary-Gonadal
LCA	Leydig Cell Area
LH	Luteinizing Hormone
LOD	Limit Of Detection
LOQ	Limit Of Quantification
MEHP	Mono(2-ethylhexyl) Phthalate
PPAR	Peroxisome Proliferation/Peroxisome Proliferator-Activated Receptor
Sox-9	SRY-related HMG box-9
SRY	Sex-determining region on the Y chromosome
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDS	Testicular dysgenesis Syndrome

Introduction

Rationales

In recent years there have been numerous reports on impaired reproductive capacity due to xenobiotics, both in wildlife, such as roach (Jobling *et al*., 2002), gulls (Helberg *et al*., 2005), alligators (Semenza *et al*., 1997) and seals (Backlin *et al*., 2003), as well as suspected effects in man (Skakkebaek, 2004). As a matter of fact, concerns about semen quality in men were raised by Danish researchers in 1992 (Carlsen *et al*., 1992), and already in the 1960s the book Silent Spring by Rachel Carson made the public aware of declining populations of certain species and the possible link to xenobiotics. One group of xenobiotic chemicals are the phthalates which are reported to be endocrine disrupting chemicals (EDCs) and cause disturbances in the reproductive organs in rats (Sjoberg *et al*., 1985; Parks *et al*., 2000). In addition, the symptoms seen with the testicular dysgenesis syndrome (TDS) in man resemble those seen in rodents after exposure to phthalates (Fisher, 2004). However, studies in other species than the rodents are few.

Background

Development of the reproductive organs in male pigs

Sexual differentiation into a male phenotype in mammals is initiated by the transcription of the SRY gene on the Y chromosome, followed by activation of the Sox-9 gene which is crucial for the development of pre-Sertoli cells in the indifferent gonad (Kanai *et al*., 2005). The pre-Sertoli cells then drive the development of the interstitial cells into Leydig cells and secrete the Müllerian Inhibiting Substance, which together with testosterone and Insulin Like Growth Factor 3 from the Leydig cells are crucial for the regression of the Müllerian ducts and the development of the phenotypic male (Nef & Parada, 2000). In the male pig embryo, the indifferent gonads can be identified by day 25 of gestation and at day 36 of gestation the gonads can be clearly identified as testicles (Hurst *et al*., 1991). Moreover, the foetal Leydig cells start to produce testosterone by day 30 of gestation (Kaminiski *et al*., 1999). In the pig the Sertoli cells proliferate both before birth, and during two distinctly different phases after birth. The first phase occurs from birth to approximately one month of age, and the second phase occurs between three and four months of age (Franca *et al.*, 2000). Furthermore, the Sertoli cell population is stable after puberty, and the number of Sertoli cells appears to determine the capacity of sperm production (Franca *et al.*, 2005). The Leydig cells follow a somewhat similar pattern of development as the Sertoli cells, with a prenatal period of proliferation, a perinatal period of proliferation and finally a period of proliferation extending from puberty into adulthood (Franca *et al.*, 2000; Franca *et al.*, 2005). Further data indicate that the size of the Leydig cells vary depending on the production of steroid hormones (Lunstra *et al.*, 1986; Franca *et al.*, 2000). In the male piglet the concentration of testosterone in plasma shows a pattern of high levels during the first few weeks after birth, and then again an increase around puberty (Figure 1)(Franca *et al.*, 2000). In boars the testes

appear to be functional around 180 days of age (Malmgren *et al.*, 1996) although development is not complete as the Leydig cells actually decrease in size after puberty (Lunstra *et al.*, 1986).

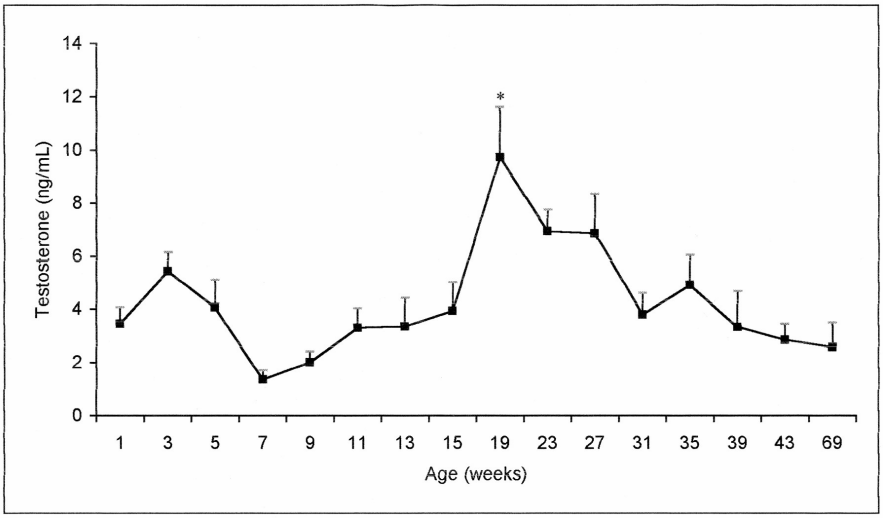

Figure 1. Testosterone in plasma in developing male pigs from birth to after puberty (Franca *et al.*, 2000). Published with permission from the *Society for the Study of Reproduction*.

Hormones and mating behaviour

Hormones are important not only for the morphological development of the reproductive tract, but also for the sexually dimorphic organization of the brain and the activation and maintenance of certain behaviours in adulthood (Kudwa *et al.*, 2005). The development of the dimorphic brain has been considered to occur around birth when testosterone produced in the testes is converted to oestradiol locally in the brain and thus effectuating masculinization and defeminization in male mammals (Kudwa *et al.*, 2005). It is still, however, uncertain whether oestrogens are needed in the brain in all species, or if the effects can be caused by testosterone directly. This has been most extensively studied in the mouse and the Japanese quail (Balthazart *et al.*, 2004). In the boar, mating behaviour is maintained in castrated animals by a combination of oestradiol and non-aromatizable androgen, but the androgen alone does not maintain the behaviour (Parrot & Booth, 1984).

On the other hand, adding to the complexity of the development of sexual behaviour, studies by Romeo (2003) and co-workers have resulted in a somewhat different hypothesis. Romeo states that not only the perinatal period is important in organization of the dimorphic brain, but also that puberty is a period of both organization and activation of already organized dimorphic sexual behaviour.

The mating behaviour in the boar has been studied previously (Parrot & Booth 1984; Tonn *et al.*, 1985; Arkins *et al.*, 1988; Thientham, 1992; Levis *et al.*, 1997)

and the whole sequence of events, which all together may take several minutes, is well outlined by the sexual behaviour index described by Levis *et al.* (1997).

Di(2-ethylhexyl) phthalate

Di(2-ethylhexyl) phthalate (DEHP) is an industrial chemical used as a plasticizer and approximately 180 000 tonnes are produced yearly (Kavlock *et al.*, 2002). Studies performed *in vitro* suggest that phthalates are oestrogenic (Blom *et al.*, 1998) but this may not fully explain the effects. For instance, the metabolite mono(2-ethylhexyl) phthalate (MEHP) inhibits the transcription of aromatase in rat granulosa cells *in vitro* (Lovekamp & Davis, 2001). In addition there appears to be a discrepancy between the effects in cell lines and in live rats; the oestrogenic effects seen in breast cancer cell lines are less obvious in rats (Hong *et al.,* 2005). *In vivo*, the toxic effects of DEHP in rats and mice have been investigated after oral or parenteral administration. In both species the organs primarily affected after exposure to DEHP are the testes and the liver, but the pituitary and kidneys are also affected. In the pituitary, there may be castration cells present after long-term exposure (David *et al.*, 2000; David *et al.*, 2001) and in female rats proteomic analysis of the pituitary revealed reduced levels of proteins involved in the release of gonadotrophins (Hirosawa *et al*., 2006). It appears that the dosages causing effects of DEHP can vary widely depending on the experimental setting in which they are tested. Some studies indicate toxic effects on the Sertoli cells in the testes in rats after a dose of around 40 mg/kg of bodyweight daily in the feed for 13 weeks (Poon *et al.*, 1997). In contrast, another study demonstrated no effects, except increased liver weights, after daily oral administration of up to 1000 mg/kg of bodyweight for nine weeks in rats (Dalgaard *et al.*, 2000). In other species, however, oral sensitivity appears to be lower. Although bodyweight in marmosets decreased after exposure to 2500 mg/kg of bodyweight, there were no organ specific effects except a small but significant increase in peroxisome volume in the liver (Kurata *et al.*, 1998). Other studies have also demonstrated lower sensitivity in monkeys than in rats (Rhodes *et al.*, 1986; Pugh *et al.*, 2000). After intravenous exposure in rats to 192 mg/kg per day by Greener *et al.* (1987) and 250 mg/kg per day by Sjoberg *et al.* (1985) both groups demonstrated effects on the liver in terms of increase in relative weight. Furthermore, Sjoberg *et al.* (1985) described effects at the electron microscopic level on the Sertoli cells.

Moreover, DEHP is a developmental toxicant in rats and mice (Kavlock *et al.*, 2002). The developmental toxicity may be mediated by some of the primary metabolites rather than by the parent compound because DEHP itself was less potent than some of the metabolites in rats (Ritter *et al.*, 1987). Effects seen in rats and mice include foetal death and developmental abnormalities in the vascular system, the reproductive system as well as the locomotor system (Kavlock *et al.*, 2002). In both mice and rats adverse effects on the fertility have been observed at doses of 110-140 mg of DEHP per kilogram of bodyweight after exposure from before mating and throughout pregnancy (Lamb *et al*., 1987; Schilling *et al.,* 1999). Arcadi *et al.* (1998) observed effects on the testes of DEHP after administration of approximately 3mg/kg of bodyweight to pregnant and lactating rats. In contrast, adult rats exposed to DEHP were sub fertile and had lesions in the

testes after administration of approximately 1100 mg/kg, but were unaffected after exposure to 290 mg/kg (Agarwal *et al.*, 1986). In addition, in studies where effects on the testes have been compared in rats of different ages, the younger rats have proved more sensitive (Sjoberg *et al.*, 1986; Dostal *et al.*, 1988); however, there are indications that this is caused by differences in kinetics (Sjoberg *et al.*, 1985). The effects seen in the testes after exposure to DEHP are probably caused by the primary metabolite MEHP (Sjoberg *et al.*, 1986; Li *et al.*, 1998).

Recent studies demonstrated anti-androgen action on the development of the reproductive tract in male rats after intra-uterine exposure (Gray *et al.*, 2000; Mylchreest *et al.*, 1998). This is in line with observations by Akingbemi *et al.* (2001) and Kim *et al.* (2003) where even low doses caused disturbances in the testosterone metabolism at different time points of development. An anti-androgenic effect has also been demonstrated, independent of testicular function, in young castrated male rats treated with both testosterone and DEHP. This anti-androgenicity was attributed to further oxidised metabolites of MEHP (Stroheker *et al.*, 2005).

Vulnerable windows in development

It has been demonstrated in different species that several chemicals may have developmental effects on an organ depending on the timing of exposure (Iguchi *et al.*, 2002). For example, exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced different degrees of impairment on the prostate or the seminal vesicle in rats, depending on whether exposure was intrauterine or postnatal or both (Lin *et al.*, 2002). In addition, developmental abnormalities are seen after exposure to phthalates during gestation (Higuchi *et al.*, 2003; Foster, 2005) but there are also negative effects on the weight of the accessory sex glands and testosterone concentrations after postnatal exposure (Higuchi *et al.*, 2003). The study by Higuchi *et al.* (2003) demonstrates the different vulnerability to dibutyl phthalate at different stages of life, with foetal life being the most sensitive, followed by adolescence as intermediately sensitive and post pubertal life as the least sensitive. Interestingly, it has been demonstrated that there is a vulnerable window for effects on sexual behaviour during the third week of post natal life, at least in rats (Feng *et al.*, 2001).

Aims

The purpose of this thesis was to increase the knowledge about endocrine disruption and investigate an *in vivo* model for evaluating putative EDCs.

The following hypotheses were tested:

- The postnatal, but prepubertal, period is a window of vulnerability of the reproductive system and the brain for exposure to DEHP

- Post natal exposure to DEHP during this putative vulnerable window of development causes delayed, long-lasting effects on the reproductive system and behaviour

In addition, the aims of this work were to:

- Challenge the generality of reproductive toxicology data generated in rats and mice
- Introduce the use of a non-rodent species in environmental research

Methodological considerations

Study designs

The data presented in this thesis are derived from three different studies; one on the kinetics of DEHP and MEHP after oral exposure in boars, one on the effects of early postnatal parenteral exposure to DEHP and one on the effects of early postnatal oral exposure to DEHP. The study described in Paper I, which deals with the kinetics of oral exposure to DEHP in the boar, was conducted on 10 piglets of approximately three months of age from different litters. Both intact and castrated animals were included in this study because the boars and barrows were easily available, and there were no indications that the presence or absence of testes affected the results obtained. Two of the animals were used for control purposes and the remaining eight were exposed to DEHP.

As presented in Paper II, the immediate and delayed effects of parenteral exposure to a low dose of DEHP or oestradiol on reproductive parameters were studied in two different experimental sets. In both of these sets, boars from four different litters were assigned to one of three groups in a split-litter design experiment. This design was used to reduce the effect of genotype (within each experimental set) on the outcome of the experiment. The immediate (acute) effects of DEHP or oestradiol were evaluated in one of the experimental sets, and the delayed effects of the same agents were evaluated in the other experimental set. To explore the oestrogenic effects of DEHP described by Blom *et al*. (1998) a group exposed to oestradiol was used in addition to the DEHP group and the control group, yielding three groups of animals.

Described in Paper III and IV, the study on the effects of oral exposure to DEHP was performed in a split-litter design where two animals from each of 10 litters were randomly assigned to either the DEHP-exposed group or the control group. Of these two animals in each of the two groups, one was randomly assigned to be euthanized immediately after the exposure period, and one was assigned to live until nine months of age (Figure 2). This design minimized the effects of genotype. Furthermore, the four groups of this study would make it possible to

make valid comparisons, not only between treatment groups, but also between different effects at different time points.

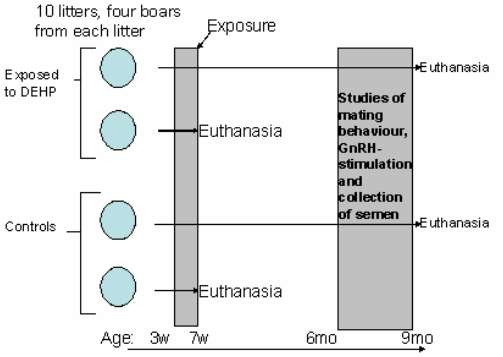

Figure 2. Schematic presentation of the study design in Paper III and IV. Each circle represents one boar in each litter

Animals

General information

All the procedures described were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden. All the boars described in Paper II, III and IV were acquired from the Lövsta experimental station of the Swedish University of Agricultural Sciences and were of mixed breed (different combinations of Swedish Landrace, Yorkshire and Hampshire). These boars were originally intended for conventional pork production, but were selected to be included in these studies based on farrowing date and the number of male piglets in the litters. Furthermore, the health status of the boars was checked by clinical examination at the initiation of the studies, as well as continually during the entire period of experimentation. The boars included in Paper I were acquired from the same experimental station, while the barrows included in that study were born at the Department of Clinical Sciences, they were offspring to sows from other studies. In all studies swine of mixed breed were used, mainly for practical reasons in terms of cost and availability. Admittedly, this could be a source of inter-group variation, but it is well compensated for by the fact that many comparisons are pair-wise and that every experimental animal had a full sibling in the control group. During the period of oral exposure some animals in both the control and the DEHP-exposed group suffered from diarrhoea, lasting from one day up to two weeks. Most of those animals showed no other signs of disease (lethargy, inappetence), but those that did were treated with trimethoprime-sulfonamide and were kept in the study. However, two boars became sick between the exposure period and the analysis of mating behaviour, and were euthanized. One of these was diagnosed with myositis (a DEHP-treated boar) and the other one was diagnosed with endocarditis after post mortal examination (a control boar). Thus, 16 boars were available for the analysis of mating behaviour, endocrinology and morphology.

Exposure of the animals

The boars described in Paper II were exposed to 50 mg/kg of DEHP in peanut oil or to 0.25 mg/kg of oestradiol benzoate by intramuscular injection. The control animals in this study received peanut oil intramuscularly. The intramuscular route was chosen because it is probably the most reliable in terms of accurate delivery in the pig. However, the availability of DEHP as well as the metabolism of DEHP after intramuscular injection is unknown. The animals were exposed from their sixth to their eleventh week of life, twice weekly. In all instances the chemicals were handled with disposable gloves, and the control animals were handled first to avoid cross contamination.

Boars described in Paper I, III and IV were administered pure DEHP with a dosing syringe in the back of the mouth; likewise, the control animals were administered water in the same manner. This method was chosen before gastric intubation because it is quicker and the risk of iatrogenic damage to the airways is probably lower. However, in a few instances there were small losses of the DEHP because some boars moved during the administration. In line with this mode of exposure, a study on the uptake and metabolism of DEHP in the young boar after oral administration was performed (Paper I). In the study on kinetics of DEHP in the boar a single dose of 1000 mg/kg was used because it was unknown what plasma concentrations to expect. Subsequently, a lower (300 mg/kg), but repeated, dose was used to study the effects on the reproductive organs (Paper III and IV). In the study on oral exposure to DEHP the effects of even earlier administration than in the study on parenteral exposure was investigated; the boars were exposed three times weekly from their fourth week to their seventh week. The animals in the control and DEHP-exposed groups were kept separate from each other, and the control group was consistently handled before the DEHP exposed group. All dosages used in these studies were kept below what was anticipated to cause clinical signs of disease, based on other studies in other species (Brevik 1976).

Analyses of DEHP and MEHP

For the study of the kinetics of DEHP and MEHP in pigs, blood samples were collected before the administration of DEHP and at 0.5, 1, 2, 4, 6, 10, 16 and 24 hours after the administration. Four pigs were then euthanized and organs (liver and testis) and tissues (fat and muscle) were sampled. From the four remaining pigs exposed to DEHP blood was obtained at 48 hours and at euthanasia after 30 days, when organs and tissues were also collected. All sampling was performed using glass tubes and metal instruments to avoid contamination with DEHP. Analysis of DEHP and its primary metabolite MEHP was performed at the Research Institute for Chromatography in Kortrijk, Belgium. A method was developed for cleaning the plasma samples from protein and to analyze the plasma for both compounds simultaneously. The plasma was analyzed by liquid chromatography-mass spectrometry, resulting in a limit of quantification (LOQ) of 0.1 mg/l and a detection limit (LOD) of 0.01 mg/l. Furthermore, the DEHP and MEHP were extracted from organs and tissue samples of 1.0 g and analyzed in a system containing a gel permeation column, followed by gas chromatography-

mass spectrometry. The resulting LOD was 10 pg and the LOQ was 20 pg. The details of chemical analysis can be found in Paper I.

Analyses of hormones

Although other methods are available for the analysis of certain hormones, such as gas chromatography (Cawood *et al*., 2005) radioimmunoassays are used in these studies because their performance is satisfactory in this context, and they are validated for use in swine. Hormonal analyses were performed at the routine laboratory at the Swedish University of Agricultural Sciences. Notwithstanding this, in the study on the effects of parenteral exposure to DEHP (Paper II) LH was analyzed at the research laboratory of one of the co-authors (Dr Madej), this time with a commercial kit specially developed for use in swine. For details, see Paper II and III.

In addition to investigating differences in hormonal concentrations in plasma occurring after exposure to DEHP, the hormonal response to a synthetic GnRH-analogue was investigated in the orally exposed boars. This approach to investigate the function of the HPG-axis has previously been described in the boar by Andersson *et al*. (1998) and a similar approach has been described in rabbits exposed to phthalates by Higuchi *et al*. (2003). In this case the dosage was adapted from Andersson *et al*. (1998) but a different system for intravenous catheterization of the boars was used. Because the need for intravenous catheterization was brief, a catheter long enough to reach the jugular vein was inserted in the auricular vein, instead of surgically inserting a catheter directly into the jugular vein.

Analyses of mating behaviour

It has been suggested that behavioural end-points are sensitive in identifying adverse effects from xenobiotics, and methods similar to those described here have been used previously in birds (Halldin *et al.*, 1999). Therefore, it was relevant to investigate effects of DEHP on the mating behaviour in boars and there are several ways to do that (Levis *et al*., 1997; Thientham, 1992). In view of this, we had to choose a protocol for evaluation of our boars and we decided to work with the system described by Arkins *et al*. (1988). Furthermore, a dummy sow was used because this would reduce any effects caused by the female on the testing (Figure 3), which may potentially be a confounder in systems where a female is used. The facilities where the boars were kept, from the start of testing, were prepared in order to ensure that the boars were unable to see when other boars were tested. In addition, the test pen was provided with a rubber floor to minimize the risk of slipping.

The protocol described by Arkins *et al*. (1988) included several parameters which we finally did not use because they were not solid enough in our hands. This was probably because the boars in our study were young and inexperienced, which in turn lead to certain events of the mating procedure sometimes occurring in the reversed sequence, or because the boars fell off the dummy sow and had to

start again. Thus, three parameters were used, which were considered robust and which worked well in our hands. These parameters were time between initial introduction to the dummy sow to first mount, time spent on the artificial sow during the mount that resulted in ejaculation and, finally, time from introduction to the end of ejaculation. In addition, the success of the mating behaviour was recorded in two different ways: the number of testing occasions needed before either mounting or ejaculation occurred, and the proportion of testing occasions resulting in mounting or ejaculation.

The time elapsed from introduction to the dummy sow to first the mount indicates the individual boar's libido, and the recording of the number of attempts needed for the initiation of a certain part of the behaviour gave us the opportunity to compare the time of sexual maturation between the treated and non-treated animals. In addition, it was possible to evaluate the mating ability of the boars by comparing the number of successful mating attempts between the groups. In all instances, the attempts to collect semen were performed by the same person on the same day, and all boars were tested in pairs where the order was randomized on every day of testing. The video recording and timing of events in the mating behaviour was always performed by the same person (the author).

Figure 3. The dummy sow used in the evaluation of mating behaviour (top). One of the boars is "courting" the dummy (bottom).

Morphology and morphometry

Since there are reports of altered organ weights after exposure to DEHP in rats (Gray *et al*., 2000) gross morphological examinations of the reproductive organs were carried out in both the studies on the effects of DEHP; the reproductive organs were inspected visually and weighed. The organ weights are presented in absolute numbers for the boars which were orally exposed to DEHP (Paper IV) although relative organ weights were used for the statistical comparisons to minimize any effects from different size of the animals. In addition to analysing the reproductive organs, certain joints in all of the boars in the study on the effects of oral exposure to DEHP were examined to evaluate whether any possible effects on mating behaviour were due to pain from osteochondrosis or osteoarthritis (Paper III). The lesions found in the joints were scored as absent, mild, moderate or severe.

Since phthalate exposure in rats and rabbits has been associated with testicular damage (Sjoberg *et al*., 1985; Higuchi *et al*., 2003) it was relevant to investigate the effects of DEHP on the histological appearance of the testes in the boar (Paper II and IV). Sections were taken from three different regions of the right testis from each boar and fixed in Bouin's fluid, as well as in paraformaldehyde, in order to perform both conventional light microscopy and immunohistochemistry. In the parenteral study (Paper II) histology was only performed on the mature boars, where there were differences in the concentration of testosterone in plasma. The methods used were based on a blinded examination of five fields of view from each of the three regions. Moreover, the magnifications used were adapted to the purpose of the examination. The presence of vacuolization of the seminiferous epithelium, loss of layers of the seminiferous epithelium and the presence of abnormal cells in the lumen of the seminiferous tubuli were examined. Similar changes were discussed by Malmgren & Larsson (1989) in boars exposed to locally increased temperature over the scrotum. In addition, the diameter of the tubuli was measured, a parameter which may be useful as an indicator of spermatogenesis, at least in rats (Lue *et al*., 2000). Furthermore, the proportion of cross-sectioned tubuli in stage VIII, as defined by Swierstra (1968), was analyzed. Yet another parameter analyzed was the area of the Leydig cells relative to the tubuli in cross sections of testis (relative LCA). To do that, a semi-automatic digital image analysis technique was developed at the Centre for Image Analysis, which is a co-venture between Uppsala University and the Swedish University of Agricultural Sciences. Unwanted regions of the images were removed manually (such as vessel, parts of the images where the tissue was broken or at the edges), then the remaining areas of the images were automatically labelled and the number of pixels representing tubuli vs. interstitium was compared. Because vimentin was used as a marker, both the Leydig cells and the Sertoli cells inside the tubuli were labelled, but on the digital images it was possible to delete the labelling inside the tubuli, which justified the use of this technique for quantification of the relative area of the Leydig cells (Figure 4). A similar relative measure was used by Oskam *et al.* (2005) in male goats. The automated analysis was used on the assumption that the technique was more repeatable between the different samples, compared to manual techniques.

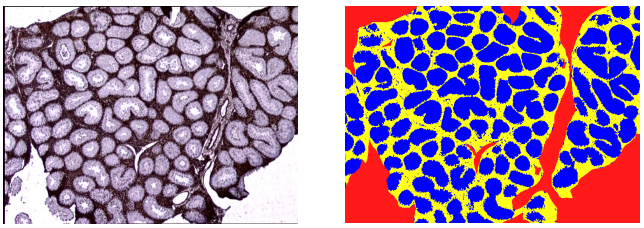

Figure 4. Example of images used for the measurement of Leydig cell area. The original image of a piece of testicle stained for vimentin (left), and the result of image processing (right) where the number of pixels can be compared.

In the study on oral DEHP, the young animals were examined for increased numbers of apoptotic cells inside the tubuli. These apoptotic cells were identified on the criteria of either having a large rounded nucleus with strong peripheral chromatin staining, or small densely stained, rounded nuclei. The purpose of this examination was to identify whether there were toxic effects of the DEHP on the Sertoli cells or the germ cells, as seen in mice after exposure to the DEHP metabolite MEHP (Giamonna *et al*., 2002). In addition, an attempt to quantify the number of Sertoli cells was introduced; reduced numbers of Sertoli cells have been observed after exposure to DEHP (Dostal *et al*., 1988). The quantification of the Sertoli cells was performed by manual counting on the computer screen of cells stained by an antibody to the murine GATA-4 zinc-finger transcription factor as previously described in the boar by McCoard *et al*. (2001).

The evaluation of the testicular morphology of the boars exposed to DEHP orally was similar to that done in the boars exposed parenterally; however, only the vacuolization of the epithelium, loss of cell layers and the presence of abnormal cells in the lumen were evaluated. The rationale for omitting the step with evaluation of the tubular diameter and the stage of spermiation was that in these boars the semen quality was continuously analyzed (Spjuth *et al*., 2006), which provides a direct measurement of the semen quality. Nevertheless, the number of Sertoli cells in the adult animals was counted in a similar way to what was done in the young animals. All histological and manual morphometrical procedures were performed by the same person (the author).

Statistical analyses

The SAS (Institute Inc., Cary, NC, USA) software package was used as the statistical tool in all studies except the kinetics study where WinNonlin Standard (Pharsight Corporation, Palo Alto, CA, USA) was used. Where multiple measurements in the same animals were performed, linear models with a factor denoting the individual animals were used in the SAS procedures GLM or MIXED. This was the case for the quantitative measurements in the behavioural studies and hormone measurements. For simple comparisons of organ weights,

paired t-tests were used in Paper IV. Where proportions were compared nonparametric tests of SAS were used and the p-values were derived from chi-square tables. This was the case for the analysis of histological, morphometrical and qualitative behavioural data. To account for small expected frequencies the Fisher's Exact test was used. The distribution of data was checked with the UNIVARIATE procedure of SAS, and when needed the data were log-transformed.

Results with comments

Paper I

In this paper the kinetics of the phthalate DEHP and the metabolite MEHP in young pigs was investigated. To some extent DEHP was found in all blood samples, even in those that should have been free from the compound. This was most likely due to contamination after sampling, during storage or handling or both. On the other hand, MEHP, the metabolite of DEHP, was only found in the samples collected after administration of DEHP, indicating that MEHP was formed *de novo* after hydrolysis of DEHP in, for instance, the intestine or the liver of the pig. An obvious peak in plasma concentration of MEHP was seen after about two hours, but because there was also a second peak, the time to maximum plasma concentration, on average, was actually eight hours.

In organs and tissues the concentrations of both DEHP and MEHP were highly variable and no reliable quantification could be obtained.

Paper II

In this paper the immediate and delayed effects of parenteral DEHP were investigated. No acute effects were observed after administration of DEHP by intramuscular injections. On the other hand, in the boars exposed to oestradiol the concentrations of both testosterone and LH were decreased during the exposure period, compared to both the control animals and the DEHP-exposed animals. When the other set of boars was examined for delayed effects at 7.5 months of age, the concentration of testosterone in plasma was higher ($p=0.005$) in the DEHP- exposed boars than in the controls. Furthermore, concentrations of LH in plasma did not differ between the groups.

At 7.5 months of age, the relative area of the Leydig cells was larger ($p=0.035$) in the DEHP-exposed boars than in the control boars. Conversely, the integrity of the seminiferous tubules of the testes did not differ between the groups.

Regarding gross morphology, immediately after exposure testes tended to be smaller ($p=0.07$) in the oestradiol-exposed boars compared with the control boars,

and seminal vesicles tended to be smaller ($p=0.05$) in the oestradiol-exposed boars compared with the controls at 7.5 months of age.

Paper III and IV

In these papers the immediate and delayed effects of oral DEHP was investigated. There were subtle differences between the control group and the DEHP group in the LH-concentration; there were variations over time in the control group, variations that were absent in the DEHP group. In the control group, at four weeks of age the LH concentration tended to be lower ($p=0.07$), and at five weeks of age it was lower ($p=0.03$) than the initial values. The concentrations of testosterone and oestradiol were lower ($p<0.001$) in both groups in the samples collected from weeks 4-7 of age compared to the initial sample from week three of age. However, the concentrations of testosterone did not differ significantly between the two treatment groups at any time, neither during the exposure nor at nine months of age.

After stimulation with GnRH at nine months of age, the concentrations of LH as well as the concentrations of testosterone were higher than baseline values within 45 minutes and two hours, respectively, in both the control group and the DEHP group. Overall, exposure to DEHP did not significantly affect the hormonal response to GnRH at nine months of age. However, the concentrations of LH tended to be lower in the DEHP-treated animals than in the control animals at 15 minutes ($p=0.1$) and 30 minutes ($p=0.06$) after the GnRH stimulation and were significantly lower 45 minutes ($p=0.04$) after the GnRH stimulation. Exposure to DEHP did not significantly affect the concentrations of testosterone at any time point.

To assess the degree of ongoing sexual maturation, boars were observed for the number of occasions needed before they displayed mounting or ejaculation. To assess libido, mating behaviour was analyzed quantitatively with respect to time requirements for different parts of the mating procedure. Finally, to assess sexual functionality, the qualitative aspects of the mating procedure were analyzed as the percentage of occasions resulting in mounting or ejaculation. However, none of the recorded parameters differed significantly between the treatment groups.

The gross morphology of the reproductive organs of boars exposed orally to DEHP did not differ from that of the control animals at seven weeks of age, *i.e.* immediately after the exposure period. In contrast, in the boars analyzed at nine months of age, the bulbourethral glands of the DEHP-exposed animals were larger ($p=0.03$) than in the control animals. The severity of joint lesions did not differ between the groups.

Finally, the microscopic evaluation of the testes of the boars revealed no differences between the two groups, neither at seven weeks of age nor at nine months of age. The only notable finding was that the only boar that showed evidence of any testicular degeneration was found in the group that had been exposed to DEHP at weaning. In this animal one section of the examined testis

was lacking spermatogenesis completely, and there was only one, or in some instances two, layers of cells which most likely were Sertoli cells and gonocytes.

Discussion

This work deals with the effects of postnatal, low doses of the endocrine disruptor DEHP on some reproductive parameters in the boar. Furthermore, effects not only immediately after exposure to DEHP, but also at later stages of life were investigated. The focus has been on long-lasting effects in the fine tuning of endocrinology and maintenance of male sex characteristics caused by exposure during the postnatal period, when the endocrine system is still under development. To evaluate the effects of DEHP, several different tools have been combined, which have been addressed in the "Methodological considerations" section of this thesis. In addition, the kinetics of DEHP in pigs had to be investigated, in order to determine whether the work was worthwhile at all.

In both studies the effects on the hypothalamic-pituitary-gonadal axis were evaluated because abnormalities in the homeostasis of this system were previously seen after exposure to DEHP in rodents (Agarwal *et al*., 1986; Akingbemi *et al*., 2001; Borch *et al*., 2004; Akingbemi *et al*., 2004). In the study on the effects of parenteral DEHP, a delayed response in the exposed animals was observed, with increased concentrations of testosterone compared to the controls at 7.5 months of age, which was 4 months after the exposure to DEHP by intramuscular injections. Increased concentrations of testosterone and derangements of the HPG- axis in the mature animal may not be of great importance to the phenotype; this would be more alarming in the developing animal. However, there may still be concerns; for example there are concerns about the use of exogenous testosterone in relation to malignancies of the prostate in men (Barqawi & Crawford, 2005). Furthermore, increased testosterone is associated with behavioural traits in some species (McGary Brougher *et al*., 2005) which may alter for example reproductive success (Kraus *et al*., 1999).

Related to the production of testosterone in the testes is the amount of Leydig cells available to produce the hormone (Johnson *et al*., 1992). It is therefore in concert with the increased testosterone concentrations to find that the DEHP-exposed group of boars, which had the highest concentration of testosterone in plasma, also had the largest area of Leydig cells relative to the tubular area. However, the concentrations of LH in peripheral plasma were not different between the DEHP-exposed group and the control group at that time. This is surprising because one would expect that high concentrations of testosterone would cause a decrease in LH or, possibly, high concentrations of testosterone would be the result of higher concentrations LH. The apparent dissociation of the feedback control of the HPG-axis has previously been seen in rats exposed to DEHP (Akingbemi *et al*., 2001).

In the other study boars were exposed to DEHP orally, an exposure route for phthalates used frequently in studies in other species (Akingbemi *et al*., 2001; Kim *et al*., 2003). However, in this study no effects were observed on the testosterone concentrations, neither during the exposure period, nor after puberty. Furthermore, plasma testosterone concentrations did not differ between the groups after stimulation with GnRH after puberty. On the other hand, LH concentrations were slightly affected. During the exposure period LH concentrations temporarily decreased in the control group, but not in DEHP-treated group at the same time. Conversely, after the GnRH stimulation LH concentrations were initially lower, but later higher, in the DEHP group compared with the control group. However, only the lower concentration was statistically significant.

In comparison, data from rats give some contradictory evidence; Borch *et al*. (2004) showed that perinatal exposure to DEHP was associated with decreased production of testosterone in the testes towards the end of pregnancy, but not at 22 days after birth. On the other hand the authors mentioned that testosterone tended to decrease at 190 days of age. Furthermore, there were indications of derangements of LH and inhibin B concentrations in the mentioned study. Interestingly, a study by Akingbemi *et al.* (2001) demonstrated differentiated effects of DEHP on the concentrations of testosterone in plasma depending on when the compound was administered in rats. Early, perinatal exposure was associated with decreased plasma concentrations, but prolonged exposure before puberty was associated with increased plasma concentrations, which is in concert with the results from the studies presented in this thesis, lending additional support to the idea of different effects after exposure during different periods of development. In the study by Akingbemi *et al.* (2001) this finding was attributed to a compensating mechanism. In addition, in the rats in that study, the concentrations of LH were high, despite the high concentrations of testosterone; therefore a disruption of the feedback control mechanism was suggested. Besides, the ability of the Leydig cells to produce testosterone was decreased in the study by Akingbemi *et al*. (2001).. In another, more recent study in rats, Akingbemi *et al.* (2004) demonstrated that exposure to a low dose of DEHP for a long period of time was also associated with increased testosterone concentrations in plasma, but decreased testosterone production per Leydig cell. On the other hand the same study demonstrated Leydig cell hyperplasia and increased oestradiol production by the Leydig cells, probably due to increased aromatase activity. In addition, inhalation of DEHP is associated with increased concentrations of testosterone in rats (Kurahashi *et al*., 2005). Adding to the complexity of the response to exposure to DEHP are further studies indicating decreases in testosterone in adult rats (Agarwal *et al.,* 1986) and decreases in both plasma testosterone concentration and aromatase activity in the testis (Kim *et al*., 2003). Furthermore, some studies demonstrate no effects on the concentrations of testosterone in adult rats after perinatal exposure (Gray *et al*., 2000).

Taken together the hormonal changes reported after administration of DEHP are at best confusing, at the worst contradictory. However, it can be concluded that the timing of the exposure is important, with regard to the effects on testosterone concentrations in plasma. The primary finding of the studies presented in this

thesis, adding to the knowledge in phthalate toxicology and developmental biology, is the fact that there are aberrations in testosterone concentrations even after postnatal exposure and a long lag phase. Possibly, the DEHP exposure deranges the delicate interplay between steroid hormones and releasing hormones during a critical time point of development. This raises some questions about the sensitivity of LH receptors in the Leydig cells and whether the receptor sensitivity is altered by exposure to endocrine disruptors during development.

In both the study with intramuscular exposure and the study with oral exposure, the effects of DEHP on the macroscopic appearance of the reproductive system as a whole entity, and the microscopic appearance of the testes were evaluated. After injection with DEHP, there were no effects observed of DEHP on the macroscopic appearance of the reproductive tract. In contrast, in the oral study, the boars exposed to DEHP had larger bulbourethral glands than the control boars at nine months of age. In the previously mentioned study by Gray *et al.* (2000), one of the effects after perinatal exposure to DEHP was decreased size of many of the reproductive organs in male rats. In addition, there is a study on the effects of post-natal (day 5-14) exposure to dibutyl phthalate on the bulbourethral glands in rats, where the glands were reported to be decreased in size, even at puberty (Kim *et al*., 2004). Furthermore, decreased accessory sex gland size has been seen in rabbits after postnatal exposure to dibutyl phthalate (Higuchi *et al*., 2003). In line with these reports Vinggaard *et al.* (2005) demonstrated decreased bulbourethral gland size after exposure to another anti-androgen during gestation and lactation in rats, without macroscopic changes in other reproductive organs.

Remarkably, in the present study the bulbourethral gland size was increased, rather than decreased, and this increase occurred a long time after exposure. Perhaps this is due to a compensatory mechanism, as suggested by Akingbemi *et al.* (2001) regarding the production of testosterone. This in turn may be evident only after puberty, when the androgens necessary for bulbourethral gland development (Cooke *et al.*, 1987) are available.

Exposure to DEHP or MEHP both *in vivo* and *in vitro* causes apoptosis of both germ cells and Sertoli cells, mediated, at least partly, by the Fas/Fas-Ligand system (Giammona *et al*., 2002; Andriana *et al*., 2004). Furthermore, high doses of DEHP cause degeneration of the spermatogenic epithelium (Sjoberg *et al*., 1985). In line with that, one of the most sensitive markers of DEHP effects on the testes was reported to be vacuolization of the Sertoli cells (Poon *et al*., 1997). In view of the above, it made sense to examine the histological appearance of the testes of the boars in these studies. The methodology was similar in the two studies described here, but in the study on parenteral exposure only the 7.5-month-old boars were included. However, no deleterious effects on the spermatogenic epithelium were observed in either of the two studies. Neither were there any differences in the number of apoptotic cells in the acutely exposed animals. In the study on oral exposure, the number of Sertoli cells per tubule was examined but no effects due to DEHP were observed. Common for previous studies in rodents where effects have been seen on the spermatogenic epithelium is that the doses

have been higher, except in the study by Poon *et al.* (1997) but the exposure period was considerably longer in that study.

There are at least three studies where the effects of DEHP on mating behaviour or mating ability have been investigated in rats (Gray *et al*., 2000; Dalgaard *et al.*, 2000; Moore *et al*., 2001) and one where the effects of dibutyl phthalate on mating were investigated in rabbits (Higuchi *et al*., 2003). There are also studies where the effects of phthalates on fertility have been studied; both Lamb *et al.* (1987) and Wine *et al.* (1997) demonstrated a reduction in fertility after continuous exposure to phthalates in mice and rats, respectively. In such studies however, not only the mating behaviour and ability to mate are evaluated, but also such factors as implantation failure and foetal losses. However, Moore *et al*. (2001) reported effects on the mating ability and sexual interest in rats after a period of exposure to DEHP. The rats in that study were exposed both during gestation and lactation. Further, Dalgaard *et al.* (2000) reported decreased mating ability in rats exposed to 10 000 mg/kg of DEHP from four weeks of age. In contrast, Gray *et al.* (2000) reported no effects on the ability to mate a female after prenatal exposure to DEHP in rats. In the present study of the effects of oral exposure to DEHP in young piglets, no effects were seen on the mating behaviour or on the mating ability after puberty. Moreover, there were no effects on the time of onset of a purposeful mating behaviour. However, as many of the boars were able to perform a purposeful mating behaviour already from the beginning of that part of the study, it would probably have been relevant to start the examinations earlier in order to find any effects on the time of sexual maturation, perhaps already at four or five months of age. Although no differences were observed between the two groups in the parameters used, it can still be considered interesting to evaluate effects of EDCs on mating behaviour in boars. The possibility of dividing the mating procedure into different phases, and the possibility of distinguishing between qualitative and quantitative parameters give the opportunity to identify deficits both in libido and in sexual function. Furthermore, the possibility of evaluating boars in the absence of a female is advantageous because there are no confounding effects induced by the behaviour of the female.

In the study of the kinetics of oral DEHP in a boar model, difficulties were encountered in measuring the concentrations of DEHP in plasma, probably due to contamination *ex vivo*. However, the kinetics were characterised for the primary metabolite MEHP, which is assumed to cause the effects on reproduction seen after DEHP exposure (Pollack *et al*., 1985). The kinetics of MEHP in the boar is somewhat different to the kinetics of the compound in the rat. The main conclusion drawn from this study was that for the same oral dosage the systemic exposure of DEHP in pigs seems to be smaller than in the rat, probably due to a smaller uptake. However, the rat seems to clear the compound from the body more effectively than the pig, as indicated by the shorter plasma half-life (Table 1).

Table 1. Comparison between pig and rat regarding some parameters in the kinetics of MEHP. The data from pigs are medians, whereas the data from the rats are means (after Sjoberg *et al*., 1985). The time to maximum concentration of MEHP in the pig was affected by a second peak in plasma concentrations; there was a primary peak at two hours after administration of DEHP

	Pig	Rat
C_{max}(mg/l)	18 (11.3-48.1)	93 (48-152)
T_{max}(h)	8 (1-24)	1-7
$t_{½\lambda}$	6.3 (5.3-9.1)	3.9 (2.4-6.8)

The studies performed with the work of this thesis focused on the issue of post natal exposure to DEHP and delayed or long-lasting effects. The effects have been detected a long time after the exposure to DEHP, which raises questions about the mechanisms causing them. Akingbemi *et al*. (2001) proposed that increased testosterone after long exposure to DEHP was due to a compensatory mechanism. Perhaps similar events, with a somewhat exaggerated compensatory mechanism, explain the effects observed in these studies on testosterone concentrations, testicular parenchyma and bulbourethral glands.

Overall, in this study the effects on the reproductive system of the boar after postnatal, but prepubertal, exposure to DEHP are limited. This may be due to several factors; one is that the total amount of DEHP or MEHP absorbed was insufficient to cause any major effects. In certain species, such as the marmoset and the cynomolgus monkey, males have been exposed to large oral doses of DEHP, without any signs of testicular toxicity. This may in fact be because of poor absorption compared to the absorption in rodents (Pugh *et al.*, 2000; Rhodes *et al.*, 1986). Another factor is that there are species differences in the response to DEHP, at least in the liver, due to the activation of Peroxisome Proliferation/Peroxisome Proliferator-Activated Receptor-α (PPAR-α) which occurs in mice and rats. It is, however, also known that testicular toxicity of DEHP occurs in PPAR-α knockout mice (Ward *et al.*, 1998). However, other PPARs such as the PPAR-γ, are known to be transactivated by MEHP (Maloney & Waxman, 1999). The role of this receptor in the testicular toxicity of DEHP does not seem to have been investigated in a knock-out mouse model. Moreover, it is also possible that the periods when the boars were exposed to DEHP were sensitive windows for the fine tuning of hormonal feedback, but not for Sertoli cell development or behaviour. Perhaps the boars were exposed too late to affect the first period of Sertoli cell development after birth and too early to affect the second period of Sertoli cell development.

Conclusions

The results of this work corroborate the hypothesis that the industrial chemical DEHP affects the reproductive endocrinology in mammals, even when exposure takes place after birth and that the effects may be seen a long time after exposure.

On the other hand, the contents of this thesis lend no support to the idea that the mating behaviour is affected by postnatal exposure to DEHP, neither were there any other detected effects on reproductive end-points, such as testicular degeneration, which may be of consequence to reproductive success.

In addition, the use of a boar model to evaluate effects on mating behaviour is promising; objective data can be generated in a highly standardized setting and analyzed both for small effects on behaviour and for overall effects on the ability to successfully transfer the gametes. Finally, in this work a non-rodent species, with a long time between birth and puberty, was used in order to evaluate the long-term effects of an EDC on reproductive parameters. The pig seems less sensitive to DEHP than rodents, which in turn may indicate that extrapolation between species is difficult and not always predictive in the field of reproductive toxicology.

Acknowledgements

The studies were carried out at the Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Swedish University of Agricultural Sciences, with the support of present and former heads of the department and its predecessors. Financial support was provided by the ReproSafe programme of the Swedish EPA.

Thanks to:

My main supervisor, Ulf Magnusson, for introducing me to science, his encouragement, his patience with my schedule and our interesting conversations about non-scientific issues.

My co-supervisors, Heriberto Rodriguez-Martinez, Björn Brunström, Leif Norrgren and Fredrik Hultén for valuable ideas, encouragement and technical assistance.

My co-authors, Karolina Törneke, Bart Tienpont, Frank David, Andrzej Madej, Patrick Karlsson, Stig Einarsson, Linda Spjuth and Kjell Andersson, for expertise in their fields.

Kjell-Åke Ahlin, Birgitta Berner, Ulrika Ehrnvall, Michael Eklund, Kjell-Ove Eklund, Elin Eriksson, Bo Fred, Helen Gille, Marete Hansen, Ulf Hermansson, Linda Jacobsson, Tom Jangby, Carola Jansson, Åsa Jansson, Dennis Larsson, Ulrika Matsson, Eva Norling, Arne Pettersson, Annika Rikberg, Steve Scott Robson, Ulla Schmidt, Karin Sellin-Wretling, Marie Sundberg and Mari Wallbring, for helping me with everything that needs to work.

The PhD-students, interns, residents and senior colleagues at SLU and at the University of Guelph, for supporting the notion of a career in research.

My friends, for good times outside work, may it be in forests, on lakes or over a meal.

My parents, brother and sisters, for many interesting discussions (about science but mostly about completely different topics) and dinners, among other things.

Ella, for keeping me company on the way to and from work.

Axel, for smiling when I come home.

Ingrid, for, among many other things, sacrificing a proper honey-moon trip.

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Svensk översättning av ”Abstract”

Målsättningen med den här avhandlingen var att öka kunskapen om störningar í hormonbalansen och om relationerna mellan påverkan före puberteten och sent uppträdande, långvariga effekter på reproduktionssystemet. Dessutom testades allmängiltigheten i de reproduktionstoxikologiska data som föreligger, då dessa till stor del tagits fram på gnagare, genom att arbetet gjordes på grisar

I två olika försök undersöktes de omedelbara och de sena effekterna för fortplantningen av plastmjukgöraren di(2-ethylhexyl) phthalate (DEHP). Dessutom undersöktes kinetiken hos DEHP och dess primära metabolit mono(2-ethylhexyl) phthalate (MEHP) hos unga grisar.

De grisar som exponerats för DEHP via injektion mellan sex och elva veckor ålder uppvisade högre koncentrationer av könshormonet testosteron i plasma och större yta av Leydigceller i testiklarna än de grisar som utgjorde kontrollgruppen. Koncentrationerna av LH, det hormon som i sin tur leder till frisättning av testosteron, var emellertid lika hos de exponerade djuren och hos kontrolldjuren, vilket antyder att exponering för DEHP tidigt i livet kan störa finjusteringen av hormonbalansen senare i livet.

Grisar som exponerats för DEHP via munnen mellan fyra och sju veckors ålder uppvisade en något annan profil av LH I plasma, jämfört med de grisar som utgjorde kontrollgrupp. De resultaten stöder i sin tur uppfattningen att DEHP stör hormonbalansen vid exponering före puberteten. I samma grupp av grisar, som exponerats via munnen, hittades skillnader i vikten hos bulbourethralkörtlarna vid nio månaders ålder, men inte vid sju veckors ålder. Dessutom jämfördes betäckningsbeteendet hos de grisar som exponerats för DEHP med kontrollgrisarna, men det gick inte att hitta några belägg för att DEHP påverkar betäckningsförmågan.

Studien av kinetik gav vid handen att grisens förmåga att absorbera MEHP efter att ha exponerats för DEHP är mindre än råttans. Detta kan i sin tur förklara att råttor i vissa fall är känsligare för verkningarna av DEHP.

De här studierna stöder hypotesen att exponering för DEHP före puberteten påverkar könshormonbalansen hos däggdjur. Däremot finns det i de här studierna inget belägg för att DEHP påverkar parningsbeteendet. Det är speciellt intressant att de effekter som ses på hormonnivåerna och reproduktionsorganen uppträder efter puberteten och inte i samband med exponeringen.