Contaminant mixtures and reproductive health: Developmental toxicity effects in rats after mixed exposure to environmentally relevant endocrine disrupting chemicals, with focus on effects in females

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Data sheet

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Summary

Background: In toxicological testing, effects of endocrine disrupters are in most cases more thoroughly investigated in males than in females. In males the hypothesis of testicular dysgenesis syndrome (TDS) proposes that there is a common origin in fetal life of the increase in frequency observed in later years in the human male population of for example incidence of boys born with hypospadias and young men with low semen quality. Furthermore, it has been observed in animal studies that exposure during fetal life to endocrine disrupters may lead to similar adverse reproductive effects. It has been proposed that a similar syndrome, called the ovarian dysgenesis syndrome (ODS), exists for females. This syndrome encompasses alterations in reproductive development caused by chemical exposure in sensitive windows of development that may result in fecundity impairments, gravid diseases, gynecological disorders or later onset adult diseases. However, experimental evidence on the effects of developmental exposure to environmentally relevant endocrine disrupting chemicals in females has been missing attention. Since chemical exposure can affect female reproductive development it is important to investigate effects in females both early and later in life.

Methods: The results, presented in this thesis, were obtained in five developmental toxicology studies. Two studies investigated mixtures of endocrine disrupting pesticides (Pestimix), two investigated mixtures of endocrine disrupting chemicals based on human exposures (Contamed), and one study tested a positive control for estrogenic effects, ethinyl estradiol (EE2). The project with the mixture of the five pesticides included two range-finding studies (collectively called Pestimix RF) and a dose response study (Pestimix DR). In the Contamed project, mixtures were modeled based on high end human intakes, and the project involved two developmental mixture studies in rats, called Contamed 1 and 2. In these studies 13 chemicals where data on *in vivo* endocrine disrupting effects and information on human exposures was available, were selected. The tested chemicals included phthalates, pesticides, UV-filters, bisphenol A, parabens and the drug paracetamol. Together the chemicals represented several modes of action with regard to endocrine disrupting mode of action. Finally, results from a dose response study on the estrogenic drug EE2 were included. In all of these studies, both male and female offspring were investigated for adverse effect of the exposure, however, the endpoints which were focused on in this thesis, were only in females. One endpoint was assessed in the pregnant dams i.e. gestational length and several endpoints were investigated in the female offspring including anogenital distance (AGD), number of nipples, onset of puberty, measurements of Anti-Müllerian hormone (AMH) and estrous cyclicity at several time point during the animals life span.

Results and discussion: Prolonged gestational length was observed in the Pestimix studies at mixture doses consisting of the pesticides at doses far below their individual no observed adverse effect level (NOAELs) for this endpoint. The effects in the mixture groups were probably caused by the azole fungicides and prochloraz which have earlier been shown to cause this effect. A longer gestational length was also observed in dams from the EE2 study at the highest dose.

AGD in female offspring was increased in both the Pestimix RF study and the DR study. Here effects were seen in the two highest mixture groups, and a similar effect was seen in females receiving epoxiconazole, prochloraz and tebuconazole, at doses equal to those included in the highest mixture dose. The effects in the mixture groups were probably caused by the azole fungicides and prochloraz, which have earlier been shown to affect AGD in females. An increased AGD was also observed in the highest dose group in the EE2 study. Puberty onset was not affected in any of the studies even in EE2 study. It is, however, recommended that in future studies recording of this endpoint is commenced at an earlier age than at pup day 27, which turned out to be too late because some of the animals had already reached puberty at that age. An increase in number of nipples was observed in the high dose group in the EE2 study. The mechanism behind this effect is not known. Estrous cyclicity was not affected in Pestimix DR but the data was not optimal as methodological difficulties caused a large proportion of the animals to become pseudopregnant. In the two Contamed studies there were no effects on estrous cyclicity in the animals at 3, 5 or 9 months of age. However, at 12 months, more animals were irregularly cycling in the highest mixture group compared with controls indicating an advance in timing of reproductive senescence. This is an important finding as premature reproductive aging is a serious effect and most studies will miss such an effect because the studies are terminated too early to observe it. There was also a significant decrease in AMH levels in 4 months old animals in the highest mixture group and the group treated with paracetamol. No effects on AMH were observed in the older animals or in the 3 months old animals in EE2 study. More works need to be done investigating if AMH can be a good marker of effects on ovarian reserve in rats. Power calculations revealed that generally the effect had to be around or above a 50% change both for effects on estrous cycle and AMH in order to be able to detect them with the used methods.

Conclusion: Female rats were affected by treatment with single EDCs or mixtures of EDCs. In all 5 studies pregnant rats and/or female offspring were affected by exposure to single chemicals or the mixture of endocrine disrupting chemicals at the investigated doses. In dams mixture effects on gestational length were reported for the first time. The affected endpoints in the female offspring were AGD, number of nipples, estrous cyclicity and AMH levels but not onset of puberty. Estrous cycle was affected only in the one-year-old female offspring and not at a younger age indicating early reproductive aging, a late effect of early exposure. The results indicate endocrine disrupters may be a contributing factor to effects of the reproductive system in women. AMH needs to be investigated more before it can be determined whether it is a marker useful in assessing effects in the ovaries after exposure to EDCs. More sensitive endpoints would be helpful when assessing effects in females and should be the focus of further studies.

Dansk resumé

Baggrund: I toksikologisk testning bliver effekter af hormonforstyrrende stoffer ofte undersøgt mere grundigt hos hanner end hos hunner. Hos hanner findes hypotesen om testikulært dysgenese syndrom (TDS), der siger at der er en fælles oprindelse i fostertilværelsen for blandt andet den øgede forekomst af drenge født med hypospadi og unge mænd med lav sædkvalitet, der er set i befolkningen i de senere år. Samtidig kan eksponering for hormonforstyrrende stoffer i fostertilværelsen hos forsøgsdyr medføre lignende effekter på forplantningsevnen og udsættelse for hormonforstyrrende stoffer mistænkes for at medvirke til de ovennævnte effekter på mennesker. Det er blevet foreslået, at et lignende syndrom, kaldet ovarie dysgenese syndrom (ODS), også findes hos hunner. Dette syndrom omfatter ligeledes ændringer af udviklingen af reproduktionssystemet blandt andet som følge af kemisk eksponering, og kan resultere i nedsat frugtbarhed, sygdomme i graviditeten, gynækologiske lidelser og sygdomme senere i livet. Der har imidlertid manglet viden om effekten af eksponering under udviklingen for miljørelevante hormonforstyrrende stoffer i hunner. Da kemisk eksponering kan påvirke den hunlige reproduktive udvikling, er det vigtigt at undersøge effekter hos hunner både tidligt og senere i livet.

Metoder: De resultater, som præsenteres i denne afhandling, er fra fem toksikologiske studier, hvor parrede Wistar hunrotter blev doseret peroralt med miljørelevante stoffer alene eller i blandinger i drægtigheds- og laktationsperioden. To studier undersøgte blandinger (miks) af hormonforstyrrende pesticider (Pestimix), to undersøgte en blanding af hormonforstyrrende kemikalier baseret på human eksponering (Contamed), og i et studie blev der testet en positiv kontrol for østrogene effekter, lægemidlet ethinylestradiol (EE2). Projektet med blandingen af de fem pesticider omfattede to pilot studier (samlet kaldet Pestimix RF) og et dosis-respons studie (Pestimix DR). I Contamed projektet blev der testet blandinger af kemikalier, som var modelleret ud fra den høje ende af hvad mennesker kan være udsat for. Projektet omfattede to rottestudier, kaldet Contamed 1 og 2. I disse studier blev 13 kemikalier blandet, hvor data om *in vivo* hormonforstyrrende effekter og information om menneskelig eksponering var til rådighed. De testede stoffer inkluderede phthalater, pesticider, UV-filtre, bisphenol A, parabener og lægemidlet paracetamol. Sammen repræsenterede kemikalierne flere hormonforstyrrende virkemåder/mekanismer. Endelig er resultaterne fra et dosis-respons forsøg med det østrogene stof EE2 inkluderet. I alle disse undersøgelser blev både han og hun-afkom undersøgt for effekter af eksponeringen, men de endpoints, der blev fokuseret på i denne afhandling, var dem der ses hos hunnerne. De undersøgte endpoints var drægtighedslængde i de drægtige hunner, og i det hunlige afkom anogenital afstand (AGD), antallet af brystvorter, alder for pubertet, målinger af anti-müllerian hormon (AMH) samt østrus cyklus på adskillige tidspunkter i løbet af dyrene levetid.

Resultater og diskussion: Forlænget drægtighedslængde blev observeret i Pestimix studierne i grupper doseret med blanding/miks af pesticider, ved doser langt under deres individuelle NOAEL for dette endpoint (”No Observed Adverse Effect Level ”), dvs. den højeste dosis af stoffet, som i dyreforsøg ikke har givet nogen skadelige effekter. Effekterne af miksen blev formentlig forårsaget af azol fungiciderne epoxiconazol

og tebuconazol samt prochloraz som tidligere har vist sig at give denne form for effekt. Længere drægtighedslængde blev også observeret i drægtige rottemødre fra EE2 studiet ved den højeste dosis.
AGD i hunafkom var øget i både Pestimix RF-studiet og DR studiet. Her blev set effekter i de to højeste miks-grupper, samt hos hunrotter, der modtog epoxiconazol, prochloraz og tebuconazol ved doser svarende til dem, der indgik i den højeste miksdosis. Effekterne i miks-grupperne blev formentlig forårsaget af epoxiconazol, tebuconazol og prochloraz, som tidligere har vist sig at påvirke AGD hos hunrotter. En øget AGD blev også observeret ved den højeste dosis i EE2 studiet. Tidspunktet for pubertetens indtræden blev ikke påvirket i nogen af de udførte forsøg, selv i EE2 studiet, som var en positiv kontrol. Det anbefales dog, at registreringen i fremtidige studier påbegyndes i en tidligere alder end ungedag 27, som viste sig at være for sent, fordi nogle dyr allerede havde nået puberteten i den alder. En stigning i antallet af brystvorter blev observeret i den høje dosisgruppe i EE2 studiet. Mekanismen bag denne virkning er ikke kendt. Østrus cyklus blev ikke påvirket i Pestimix DR, men data var ikke optimale da metodologiske vanskeligheder forårsagede at en stor del af dyrene blev pseudogravide. I de to Contamed studier var der ingen effekt på østrus cyklus i dyrene i 3, 5 eller 9-10 måneders alderen. Men efter 12 måneder havde flere dyr uregelmæssig cyklus i den højeste miksdosis-gruppe sammenlignet med kontroller. Dette indikerer en tidligere reproduktiv ældning (menopause/overgangsalder i mennesker). Dette er et vigtigt resultat, da for tidlig menopause er en alvorlig effekt, og de fleste studier vil overse sådan en effekt, fordi studierne afsluttes for tidligt til at observere den. Der var også et signifikant fald i AMH niveauer i 4 måneder gamle dyr i højeste miksdosis-gruppe og i gruppen behandlet med paracetamol. Der blev hverken observeret effekt på AMH i de ældre dyr eller i de 3 måneder gamle dyr i EE2 studiet. Der skal mere viden til for at afgøre om AMH kan være en god markør for effekter af hormonforstyrrende stoffer på ovariefollikelreserven hos rotter. Power beregninger afslørede at generelt kan man måle omkring en 50% ændring både for effekter på østrus cyklus og AMH.
Konklusion: Hunrotter viste sig at være følsomme for eksponering med blandinger af hormonforstyrrende stoffer. I alle 5 studier blev drægtige rotter og /eller hunligt afkom påvirket ved eksponering for de enkelte kemikalier eller miks af hormonforstyrrende kemikalier ved de undersøgte doser. For første gang er der rapporteret mixeffekter på gestationslængden hos drægtige rotter. I afkommet blev der set effekter på AGD, antal brystvorter, østrus cyklus og AMH niveauer, men ikke tidspunkt for pubertet. Effekter på østrus cyklus blev kun set hos de et år gamle rotter og er et tegn på sene effekter af tidlig eksponering. Resultaterne indikerer at hormonforstyrrende stoffer kan være medvirkende faktor til effekter i reproduktionssystemet hos voksne kvinder og ufødte pigebørn. AMH skal undersøges bedre for at vurdere om det er en god markør for effekter af hormonforstyrrende stoffers effekt på ovariefollikelreserven. Mere følsomme endpoints ville være nyttige ved vurderingen af effekter hos hunner og bør være i fokus i fremtidigt arbejde.

Table of Content

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Abbreviations

AGD	Anogenital distance
AGDI	Anogenital distance index
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
AR	Androgen receptor
BPA	bisphenol A
Bw	Body weight
D	Diestrus
DA	Dose addition
DBP	dibutyl phthalate
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DEHP	Di(2-ethylhexyl)phthalate
DES	diethylstilbestrol
DR	Dose response
E	Estrus
EE2	Ethinyl Estradiol
Epoxi	Epoxiconazole
GD	Gestation Day
GnRH	Gonadotropin- Releasing Hormone
HPO axis	hypothalamic-pituitary-ovarian axis
HPTE	2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane
IA	Independent action
LH	Luteinizing hormone
LOAEL	Lowest observed adverse effect level
M	Metestrus
Manz	mancozeb
4-MBC	4-methyl-benzylidene camphor
MIS	Müllerian inhibiting substance
MXC	Methoxychlor
NA	Not applicable
NOAEL	No observed adverse effect level
NTP	National toxicology program
OECD	Organisation for Economic Co-operation and Development
OMC	Octyl methoxycinnamate

P	proestrus
p,pDDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
PCB	3,4,3',4'-tetrachlorobiphenyl
PD	Pup day
PM	paracetamol
PND	Post Natal Day
Prchl	prochloraz
Procy	procymidone
RF	Range finding
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Tebu	tebuconazole
US EPA	United States Environmental Protection Agency
VIC	Vaginal Impedance Checker
VO	Vaginal Opening

List of papers

Paper I. Jacobsen PR, Christiansen S, Boberg J, Nellemann C, Hass U. Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats. Int J Androl. 2010 Apr;33(2):434-42.

Paper II. Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A, Clemmensen LH, Axelstad M.. Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod Toxicol. 2012 Sep;34(2):261-74. Epub 2012 May 29.

Paper III. Jacobsen PR, Axelstad M, Boberg J, Isling LK, Christiansen S, Mandrup KR, Berthelsen LO, Vinggaard AM, Hass U. Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod Toxicol. 2012 Sep;34(2):237-50. Epub 2012 Jun 4.

Paper IV. Christiansen S, Kortenkamp A, Axelstad M, Boberg J, Scholze M, Jacobsen PR, Faust M, Lichtensteiger W, Schlumpf M, Burdorf A, Hass U.. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. Int J Androl. 2012 Jun;35(3):303-16. doi: 10.1111/j.1365-2605.2011.01242.x. Epub 2012 Feb 28.

Paper V. Mandrup KR, Jacobsen PR, Isling LK, Axelstad M, Dreisig K, Hadrup N, Vinggaard AM, Hass U, Boberg J. Effects of perinatal ethinyl estradiol exposure in Wistar rats. (submitted to Reproductive Toxicology).

1 Introduction

Endocrine disrupting chemicals/compounds (EDCs) have been defined by The World Health Organisation (WHO) and the International Programme for Chemical Safety (IPCS) as follows (World Health Organization (WHO) 2002):

> An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations .

EDCs have been speculated to contribute to a rise in different types of reproductive problems the last couple of decades (Skakkebaek et al. 2001). Main center of attention has been on effects in males of developmental exposure to EDCs, e.g. decreasing sperm counts, increase in incidence of hypospadias and cryptorchidism in human and animal models, which have been extensively investigated (Christiansen et al. 2008, Gray et al. 2001, Main et al. 2009, Main et al. 2010, Noriega et al. 2005, Wilson et al. 2008, Wohlfahrt-Veje et al. 2009). However, in recent years more attention have been turned toward female health and exposure to environmental chemicals has been proposed to contribute to several gynecologic pathologies, especially when exposures occur during critical windows in development (Caserta et al. 2008). A similar evaluation as has been conducted in males encompassing clinical trends and studies, laboratory animal studies, and comparative biology data collected from wildlife has not been done in females to nearly the same extent (Caserta et al. 2008, Crain et al. 2008). Thus, more research in this area is warranted.

Exposure to EDCs in adulthood may have very different consequences than exposure during development. The concept described by Barker as ”the fetal basis of adult disease” was extended by Diamanti-Kandarakis and co-workers to “the developmental basis of adult disease” because some organs keep undergoing development in early postnatal life (Barker 2003, Diamanti-Kandarakis et al. 2009).What this concept describes is that there can be a lag between time of exposure and the manifestation of a disorder in puberty, adulthood or during aging. A classical example of endocrine disruption occurring *in utero* leading to adult disease in females is the case of occurrence of rare gynecological neoplasms, vaginal adenosis and structural abnormalities present at birth such as cervical hoods, ridges, and T-shaped uterus in the daughters of women treated with the estrogenic pharmaceutical diethylstilbestrol (DES) during their pregnancy (McLachlan et al. 2006, Mittendorf 1995). In males there is a hypothesis named testicular dysgenesis syndrome (TDS). TDS is based on observations made in epidemiological and partly in animal studies that hypospadia, cryptorchidism,

testicular cancer and possibly lowered semen quality may be associated with each other as different manifestations of disturbed prenatal testicular development. Endocrine disrupters may contribute to an observed rise of these symptoms in men in later years (Skakkebaek et al. 2001, Wohlfahrt-Veje et al. 2009). It has been proposed that a similar syndrome exists for females, which is called ovarian dysgenesis syndrome (ODS). This syndrome encompasses alterations in reproductive development caused by chemical exposure in sensitive windows of development that may result in or contribute to fecundity impairments, gravid diseases, gynecological disorders or later onset adult diseases but is less well described and defined as TDS (Buck Louis et al. 2011, Buck Louis et al. 2006, Fowler et al. 2012). Adverse effects on development may result from either chemical exposure causing structural birth defects or changes in epigenetic programming during development but for the most part of the disorders the involved molecular mechanisms has yet to be elucidated (Buck Louis et al. 2011, Newbold and Kinyamu 2010).

1.1 Aim and objectives of this PhD thesis

Presently, effects of endocrine disrupters are more thoroughly investigated in males than females, and more research on effects in females are warranted. Furthermore, experimental evidence has been missing on the effects of combined developmental exposure to environmentally relevant endocrine disrupting chemicals with antiandrogenic or estrogenic modes of action. Antiandrogens are not generally expected to cause effects on estrogen-sensitive endpoints but may potentially augment the effects of estrogens. Consequently, it is relevant to study combined effects of chemicals with antiandrogenic or estrogenic modes of action.

The PhD thesis includes data from five studies, four mixture studies and a study with the positive estrogenic control, ethinyl estradiol.

Overall, the aim of the PhD thesis was to investigate effects on sexual development in female rats perinatally exposed to single EDCs or mixtures of EDCs and on gestational length of the dams. The objectives were to address the following hypotheses:

- Perinatal exposure to environmentally relevant EDCs can affect gestation length in dams and hormone-sensitive endpoints in female offspring
- The endpoint estrous cyclicity is sensitive to exposure to these environmentally relevant EDCs, and effects of exposure can be detected on estrous cycle in aging rats
- Measurement of plasma levels of anti-müllerian hormone (AMH) is a useful marker of early reproductive senescence
- Effects on hormone-sensitive endpoints in females will be observed with an estrogenic positive control compound.

This thesis will focus on one endpoint in dams directly exposed to mixtures or single chemicals with endocrine disrupting properties i.e. gestational length and on several endpoints in the female offspring exposed during gestation and lactation, including effects on anogenital distance (AGD), number of nipples, puberty onset, levels of Anti-Müllerian hormone (AMH), and estrous cyclicity at several ages. Furthermore, some method related considerations will be discussed. The following sections will address the main points of the female differentiation, the endpoints which are the focus of this thesis and a short introduction to mixtures.

2 Background

2.1 Sexual differentiation focusing on females

This section will cover the overall lines of sexual development in the female as this is a sensitive and complex process, in which a developing fetus may be vulnerable to exposure to endocrine disruptors (Gray 1998).

In mammals early in development, the gonads are indifferent but either being chromosomally XX (female) or XY (male). Development of the gonad in a male direction is dependent on the gonadal expression of the gene S*ry* (sex determining region Y) on the Y chromosome. In the absence of the S*ry* gene the gonad develops as an ovary. The S*ry* gene codes for a transcription factor that initiates a cascade of genes affecting the indifferent gonad to develop in a male direction. Many genes have also been identified as being involved in the development of the indifferent gonad into the ovary (Goldman and Cooper 2010).

Figure 1 shows a sexually undifferentiated gonad with Müllerian ducts and Wolffian ducts. Anti Müllerian Hormone (AMH) produced by Sertoli cells in the embryonic testes induces regression of the Müllerian ducts and causes the Wolffian ducts to differentiate into epididymis and vas deferens tissues. In the absence of the hormones produced by the embryonic testes (testosterone and AMH), the Wolffian ducts degenerate and the Müllerian ducts develop into female structures (Goldman and Cooper 2010).

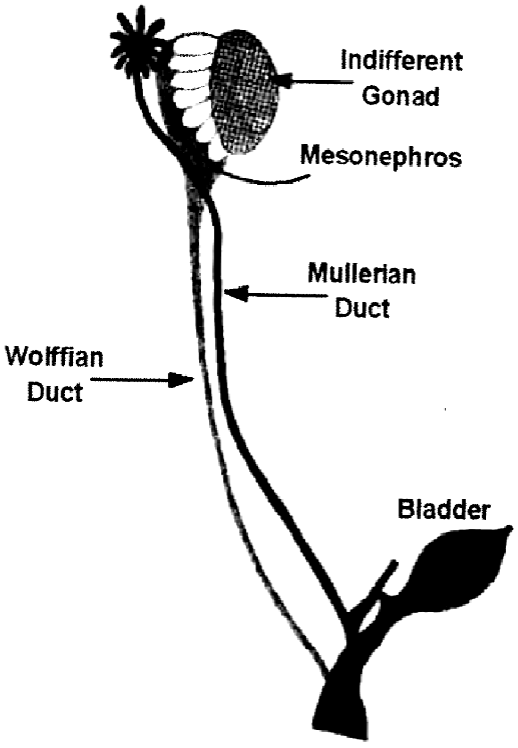


Figure 1. Modified from (Goldman and Cooper 2010). The figure represents the primitive gonad with the Müllerian and Wollfian ducts in the sexually undifferentiated embryo.

As mentioned previously, it has been hypothesized that chemical exposure during the vulnerable period of fetal and early postnatal development may lead or contribute to effects observed later in life, a phenomenon in females referred to by some researchers as the ovarian dysgenesis syndrome (Buck Louis et al. 2011, Buck Louis et al. 2006, Fowler et al. 2012).

Two key events in ovarian development are follicle assembly and follicle transition from primordial to primary follicle (initial recruitment). These processes, which are not yet fully understood, are thought to involve regulation with endogenous growth factors, steroids, cytokines, and a less certain role for gonadotropins (though, gonadotropins are required for the later maturation of preantral follicles into the more mature phases of development) (Billiar et al. 2003, Eppig and O'Brien 1996, Kezele and Skinner 2003, Roy and Albee 2000, Skinner 2005, Uzumcu and Zachow 2007, Wang and Roy 2006). These processes directly affect the number of oocytes available to a female throughout her reproductive life (Kezele and Skinner 2003, Skinner 2005, Uzumcu and Zachow 2007).

Critical steps in this process may be disturbed by chemical exposure to endocrine disrupters affecting reproductive function or the duration of reproductive lifespan (Abbott et al. 2006, Skinner 2005, Uzumcu and Zachow 2007). Indeed, as it has been shown for a number of endocrine disrupters that different stages of folliculogenesis can be affected by some endocrine disrupting chemicals, and some examples of chemicals that affect initial recruitment are shown in figure 2. Methoxychlor (MXC) blocks pre-antral to antral transition (Chapin et al. 1997, Uzumcu et al. 2006) (effects *in vivo*), bisphenol A (BPA) affects follicular assembly and induces multi-oocyte follicles (*in vivo*) (Suzuki et al. 2002) and also DES affects follicular assembly and induces multioocyte follicles (*in vivo* and *in vitro*)(Iguchi et al. 1990).

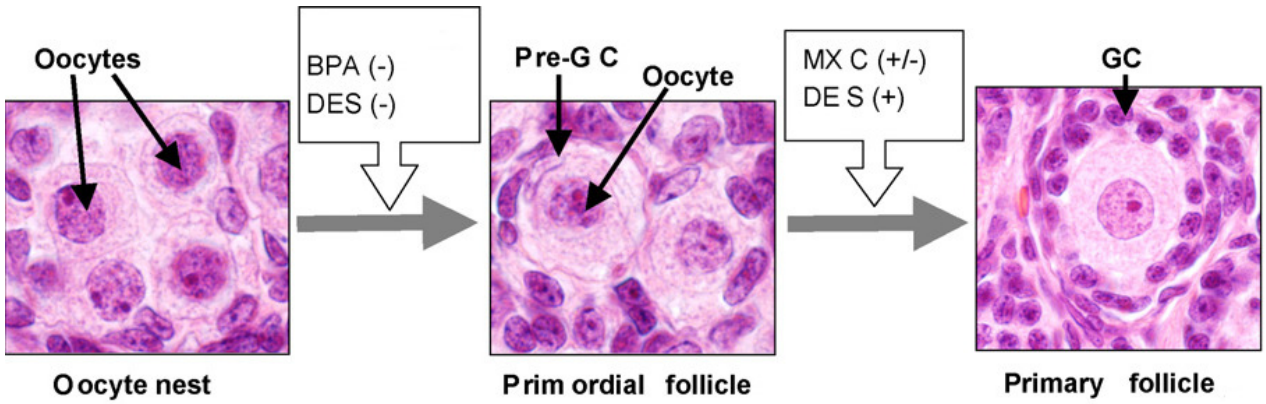


Figure 2. An overview of ovarian folliculogenesis modified from (Uzumcu and Zachow 2007). Following apoptosis of some oocytes within the oocyte nests the primordial follicles are formed. The remaining oocytes are then surrounded by squamous precursor-type granulosa cells (pre-GC). The (+) and (−) denotes that the chemical has stimulatory or inhibitory effects on some of the processes taking place at that stage. BPA=bisphenol A, MXC=Methoxychlor, DES= diethylstilbestrol.

2.2 Gestational length

In this thesis as well as in reproductive toxicology studies in general, gestational length is an important endpoint. Significantly longer or shorter gestation length may be caused by malfunction of the normal mechanism for parturition. Shortening of gestational length can lead to adverse outcomes of pregnancy such as decreased birth weight and survival of the offspring. Prolongation of gestational length could result in death or impairment of offspring if difficulty in parturition (dystocia) occurs. Dystocia is a maternal health threat for humans as well as experimental animals. Gestation length can easily be determined in experimental animals from data on day of mating and day of parturition (OECD 2008, U.S. EPA 1996).

The exact physiological stimulus for parturition is not fully understood (Young 2001). At near-term relaxation of the pubic symphysis begins (by day 17 of gestation in rats) and is complete prior to parturition (on GD22-23 in rats). The hormone relaxin, produced by the corpus luteum during the second half of pregnancy, is involved in the cervical extensibility as well as relaxation of the pubic symphysis in the rat (Samuel et al. 1996). Relaxin is also involved in remodeling of the cervix in humans along with other factors such as prostaglandins and metalloproteinkinases (Basavarajappa et al. 2010). Progesterone and estrogen are, among other signaling compounds, involved in the contractions of the myometrium of the uterus (Basavarajappa et al. 2010). In rats progesterone levels decrease prior to onset of labor which is caused by regression of the corpus luteum, which in the rat is the main site of progesterone production during pregnancy (Challis and Lye 1994, Mesiano and Welsh 2007, Young 2001). In humans, circulating progesterone levels primarily originates from the placenta after the sixth to nine week of gestation and does not seem to decrease during labor and delivery but is thought to involve a form of progesterone withdrawal that does not involve a decrease in the circulating levels (Challis and Lye 1994, Mesiano and Welsh 2007).
Progesterone's role in maintaining pregnancy and promote myometrial relaxation seems to be a conserved trait in viviparous species (Mesiano and Welsh 2007). In rats, progesterone blocks myometrial activity and withdrawal due to luteolysis leads to onset of labor and delivery but other factors that stimulate myometrial activity such as estrogen, oxytocin and prostaglandins are also implicated in the process of giving birth (Basavarajappa et al. 2010, Kobayashi et al. 1999). In humans, progesterone also inhibits contractions while estrogen induces myometrial contractions and stimulates production of prostaglandins, oxytocin, contractile-associated proteins, and estrogen receptor α (Basavarajappa et al. 2010). In most species, including the rat, estrogen has a stimulatory effect on myometrial contractility that is mediated by an increase in circulating estrogen levels prior to onset of labor and is coordinated with systemic progesterone withdrawal. In humans, however, circulating estrogens increase at around mid-gestation and continue to rise gradually until birth (Mesiano and Welsh 2007). In the last phases of parturition of both rodents and humans, prostaglandins are involved in the strong contractions of the myometrial of the uterus to eventually ensure delivery (Basavarajappa et al. 2010).

Chemicals that interfere with the processes of parturition could lead to prolonged or shortened gestational length depending on the mechanism being disrupted. There are several examples of chemical compounds which prolong gestational length in laboratory animals such as the industrial chemical 3,4,3',4'-tetrachlorobiphenyl (PCB), zeranol (food additive for cattle in USA) (Rands et al. 1982) and the pesticides prochloraz (Noriega et al. 2005, Vinggaard et al. 2005), epoxiconazole and tebuconazole (Taxvig et al. 2007). The pharmaceutical finasteride has been observed to reduce gestation length in rats (Paris et al. 2011) and perinatal 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure in mice has been shown to cause premature parturition in the offspring when exposed *in utero* (Ding et al. 2011). In humans, the maternal serum levels of 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,pDDE) the metabolite of the pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (DDT) has in some studies been associated with preterm birth and cord serum levels from premature newborns of hexachlorobenzene and DDE was higher in preterm born children compared to babies born to term (Longnecker et al. 2001, Ribas-Fito et al. 2002). However, another study failed to find a significant association between exposure to p,p'-DDE and preterm birth (Torres-Arreola et al. 2003).

2.3 Anogenital distance and nipple retention

Two very sensitive endpoints that have been widely used as biomarkers when investigating chemicals for endocrine disrupting properties in rats are anogenital distance (AGD) and nipple retention (NR).

Anogenital distance in new born rat pups is defined as the distance from the anus to the genital bud (see Figure 3). AGD is a sexually dimorphic secondary sex characteristic in many mammalian species including humans (Salazar-Martinez et al. 2004) and is dependent on prenatal exposure to androgens, which stimulate the growth of the perineum (Bowman et al. 2003, Clemens et al. 1978, Ostby and Gray 2004, Zehr et al. 2001). Thus, AGD is longer in males than in females. As a rule of thumb the AGD measure in male rats is about two fold greater than the distance in female rats and the same applies for humans (Clemens et al. 1978, Ostby and Gray 2004, Salazar-Martinez et al. 2004). The endpoint is usually measured shortly after birth in rodents and correction for body weight has to be performed in the analysis of the data (Gallavan et al. 1999).

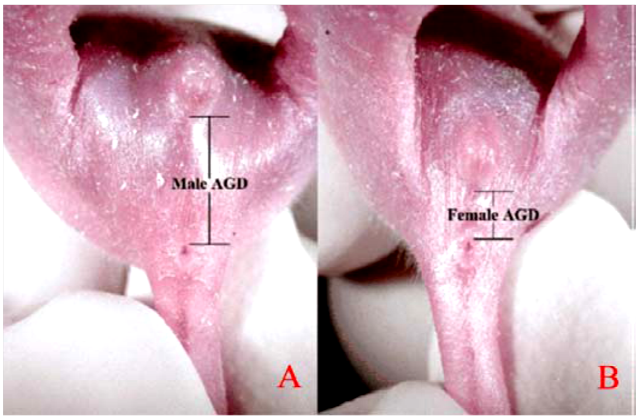


Figure 3. AGD in a normal male rat (A) and a female rat (B) pictures modified from (Ostby and Gray 2004).

Mammary glands are developed in both male and female fetal rats and adult female rats most often have 12 nipples. However, the male nipples are regressed prenatally due to the action of androgen (in rats dihydrotestosterone) in the tissue leaving only nipple anlagen and rudimentary mammary glands. In males, these are normally not visible to the naked eye (or only very few) when pups are 13 days old, which is the age nipples are often assessed in rats (Barlow et al. 2004, Hotchkiss et al. 2004, Imperato-McGinley et al. 1986, Kratochwil 1977, Ostby and Gray 2004). Figure 4 A and B shows the anlagen of a female rat and a male rat exposed to antiandrogen. Nipples in females are more pronounced than in males exposed to antiandrogens but as can be seen in the figure, they are visible to the naked eye in both sexes (Figure 4). Since these tissues are androgen dependent, anogenital distance and nipple retention are most often used as endpoints in assessing effects of endocrine disruptors (most often anti-androgens) on the male reproductive system and numerous chemicals has been shown to affect AGD and nipple retention in male rats (Barlow et al. 2004, Boberg et al. 2011, Christiansen et al. 2010, Gray et al. 1999, Noriega et al. 2005, Ostby et al. 1999). Generally, both AGD and NR are two very sensitive and non-invasive endpoints, when investigating effects of anti-androgenic compounds administered during the critical period of male sexual development (Christiansen et al. 2010).

There are, however, also examples where AGD in female offspring has been affected by a treatment. A longer AGD than in control animals may be a consequence of androgen exposure during development (Ostby and Gray 2004). Exposure to estrogenic acting compounds like ethinyl estradiol and genistein have also been shown to alter AGD in female rats, however, sometimes increased AGD is reported and sometimes shortened AGD is reported with exposure to these compounds (Casanova et al. 1999, Delclos et al. 2009, Levy et al. 1995, National Toxicology Program 2010). Effects on AGD of female rat offspring manifested as increased AGD have also been observed after *in utero* exposure to some fungicides like prochloraz and epoxiconazole (Laier et al. 2006, Taxvig et al. 2007). There are a few reports of significant effects on the number of nipples in rodent females in the open literature and they are reporting androgen exposure during development

resulting in a lower number of nipples (Hotchkiss et al. 2007, Wolf et al. 2002, Wolf et al. 2004). No reports in the open literature have been found where more than the normal number of nipples is observed in female rats.

2.3.1 Relevance to humans

In rats AGD and nipple retention has been shown to be highly predictive of adverse effects of the male reproductive system including increased incidence of hypospadias, decreased testosterone and altered reproductive organ weight changes (Bowman et al. 2003, Christiansen et al. 2008, Macleod et al. 2010, van den Driesche et al. 2011, Welsh et al. 2008). Measurement of AGD in men has shown some interesting results so far as AGD have been shown to correlate with changes in semen parameters (Eisenberg et al. 2011, Mendiola et al. 2011). A correlation between decreased AGD and decreased testosterone level has furthermore been observed in men (Eisenberg et al. 2012). There has previously been reported a strong inverse association between prenatal phthalate exposure (particularly the anti-androgenic di-2-ethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP)) and shorter male AGD in human infants (Swan et al. 2005, Swan 2008). Nipple retention or number of nipples are not observed effect in humans but the relevance of this endpoint is tied to the cause of this effect, which is the ability of chemicals to impair androgen action during development. No papers have been found reporting effects on AGD in girls or women.

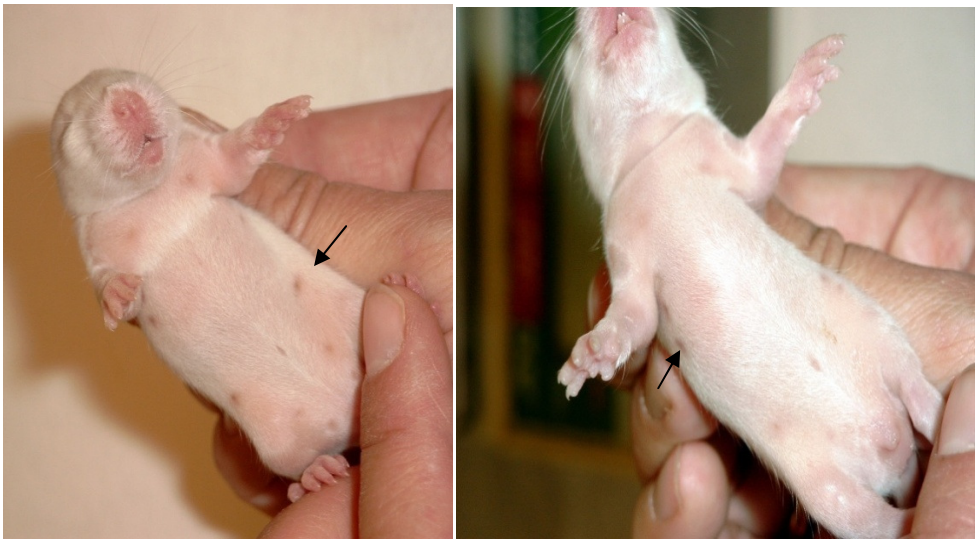


Figure 4 (A), female control rat with 12 nipples present (B) a male rat exposed to anti-androgen. Arrows points to the dark areas, which are the nipples or "dark areas" counted (photo: Bo Herbst).

2.4 Onset of puberty.

Onset of puberty has been evaluated as an endpoint in toxicological tests for many years (Gray et al. 1988, Gray et al. 1989, Gray et al. 2004, Lewis et al. 2002). In female rats onset of puberty is often measured as the time of vaginal opening (VO) also called vaginal patency. After the canalization of the vagina the vaginal orifice remains covered by a septum in female rodents. The day when the septum is no longer present is the

day of VO and this is readily determined as can be seen from Figure 5. VO generally occurs with or just before first ovulation and is a reliable marker for onset of puberty in rats (Parker and Mahesh 1976) and commonly takes place between postnatal days 30 and 37 (Clark 1999, Goldman et al. 2000).

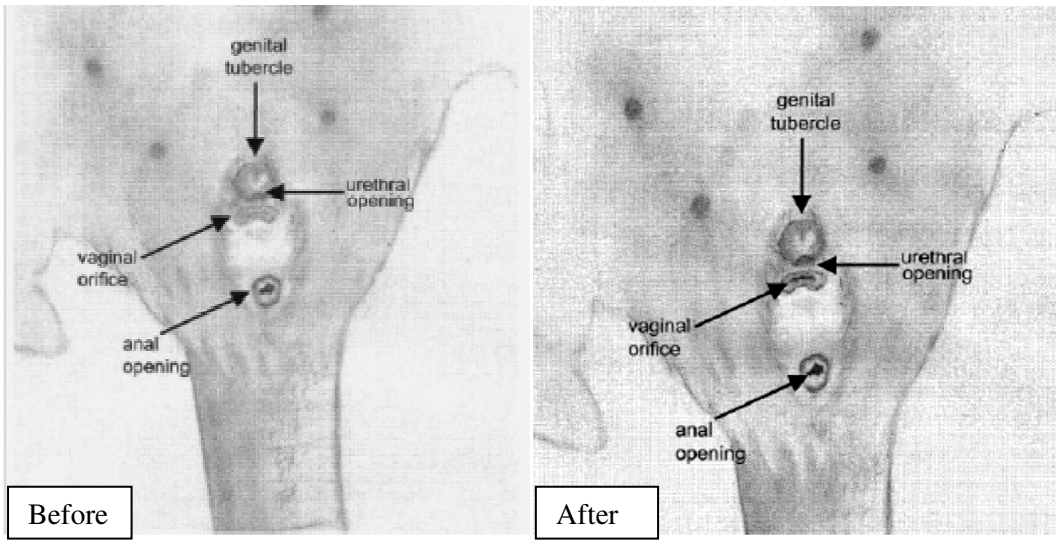


Figure 5. The figure shows the area around the anal opening and the genital tubercle of a female rat before and after the septum has disappeared. Modified from (Lewis et al. 2002)

Puberty is a transitional process, characterized as a cascade of neuroendocrine events leading to maturation of the reproductive system and is considered to be complete in the female when regular ovulatory cycles are established (Crain et al. 2008, Urbanski and Ojeda 1987). In the female rat puberty encompasses vaginal opening (VO) and first estrous (ovulation) which are both used as markers of puberty onset (Goldman et al. 2000, Gray et al. 1988, Gray et al. 1989). While the exact factors that trigger onset of puberty are not fully understood, onset of puberty in the female rat seems to be dependent both on genetic and environmental factors including but not limited to nutrition, light and stress (Rivest 1991) but it can also be affected by chemical exposure (Goldman et al. 2000).

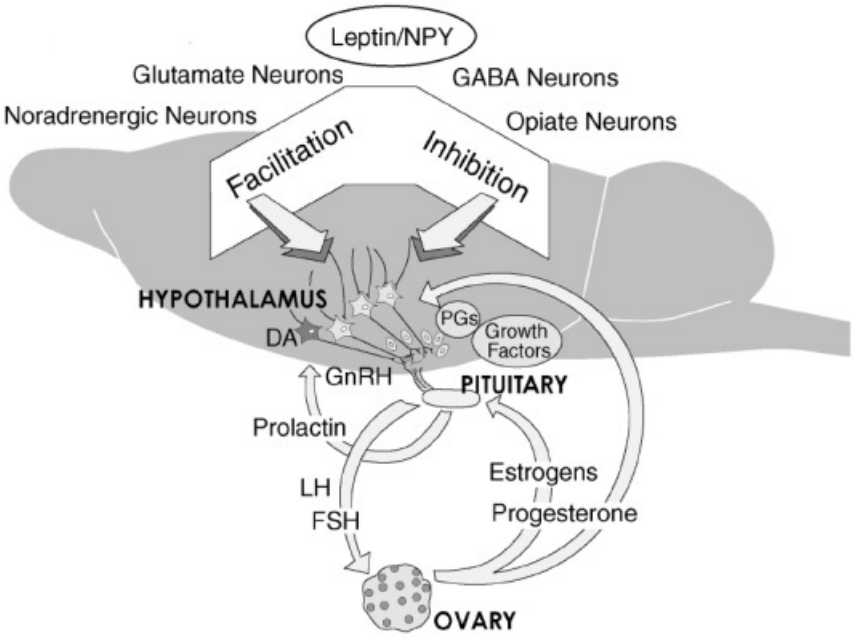


Figure 6. A generalized figure of the principal factors involved in the hypothalamic-pituitary-ovarian axis during onset of puberty in the female rat. For further explanation see the text (Goldman et al. 2000).

The pubertal events are a consequence of progressive functional shifts in the hypothalamic-pituitary-ovarian axis (Ojeda and Urbanski 1994). A generalized figure of the principal factors involved in the hypothalamic-pituitary-ovarian axis during onset of puberty in the female rat is seen in Figure 6 (Goldman 2000). When puberty is imminent the hypothalamus begins to secrete Gonadotropin- Releasing Hormone (GnRH) in pulses which in turn stimulate the pituitary to release pulses of LH and FSH in to the circulation (Crain et al. 2008). This phenomenon is not well understood and is referred to as the GnRH pulse generator. A number of neurotransmitter systems participate in the release of GnRH, some stimulating e.g. (norepinephrine (Noradrenergic) and glutamate neurons) others inhibiting (e.g. opiate neurons and GABA neurons) release of GnRH (Goldman et al. 2000). For an individual transmitter system puberty can be a period of transformation of function. For example γ-aminobutyric acid (GABA) is stimulating gonadotropin release (probably through GnRH) during prepuberty but inhibits gonadotropin release in peripubertal animals. The ovary responds to the circulating gonadotropins and excretes estrogen and progesterone which in turn exerts feed back to the hypothalamus and pituitary affecting the release of GnRH and gonadotropins, respectively (Goldman et al. 2000). In three weeks old rats estradiol excerts negative feedback on hypothalamus and the pituitary, but a shift in the feedback happens in four week old rats and the hypothalamus and the pituitary start reacting positively to estradiol feedback (Andrews and Ojeda 1981, Andrews et al. 1981, Goldman et al. 2000). As the system becomes more sensitive and the rat matures this feedback process leads up to the occurrence of VO (estrogen dependant) and a preovulatory surge of LH (Ojeda and Urbanski 1994, Ramirez and Sawyer 1965). Prostaglandins and kisspeptin are also involved in modulating the GnRH secretion (Ojeda et al. 1986,

Rage et al. 1997, Roa et al. 2008, Roa et al. 2009, Tena-Sempere 2010). Furthermore, data suggests that many other compounds like e.g. leptin, neuropeptid Y, growth factors and thyroid hormones also play a role in puberty onset (Cheung et al. 1997, Goldman et al. 2000, Gruaz et al. 1993, Ma et al. 1992, Minami and Sarkar 1992, Tamura et al. 1998).

2.4.1 Types of chemicals that affect VO in the female rat

The process of sexual maturation is as mentioned a complex neuroendocrine process and chemical insult that interferes with any of the involved processes may result in either an advance or a delayed onset of puberty for some chemicals even from exposure in utero and during lactation (Goldman et al. 2000, Grande et al. 2007, Gray and Ostby 1995, Si et al. 2011, Urbanski and Ojeda 1987, Wang et al. 2005). Delay in onset of puberty can be a non-specific effect of marked general toxicity or a specific effect of a chemical, however, a lowered body weight gain is not always indicative of a delay in sexual maturation as with some estrogenic compounds which cause lower body weight but accelerated VO (Delclos et al. 2009, Goldman et al. 2000, Gray et al. 1989).

Examples of types of chemicals that can advance onset of puberty in rats are chemicals with suggested estrogenic mechanism of action. For example, ethinyl estradiol advanced puberty in female rat offspring after dietary exposure from GD7 and through lactation and after weaning (National Toxicology Program 2010). Also methoxychlor or its metabolite 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) acts both as an estrogen receptor agonist and androgen receptor antagonist and advances puberty when dosed by gavage from weaning to adult (Bulger et al. 1978, Gray et al. 1989, Maness et al. 1998). Furthermore, the testosterone synthesis inhibitor DEHP administered by inhalation PND22-84 advanced puberty in female rats (Ma et al. 2006).

Many types of chemicals seem to be able to cause a delay of VO in studies on rats. These includes, but are not limited to, treatment with the ah-receptor agonist TCDD exposed GD15 by gavage (Gray and Ostby 1995), aromatase inhibitor (Fadrozole, PND21-40 by gavage (Marty et al. 1999)) and atrazine (reduces LH and prolactin) PND22-41 orally by gavage (Laws et al. 2000b). These delays were not attributable to a lower body weight.

2.4.2 Human relevance

Over a period of about 100 years until the mid twentieth century a decline in age of onset of puberty in girls has been observed. According to Parent et al (2003) the general improvement of living conditions has probably been the major explanation for the decline. Thereafter age at onset of puberty seems to have stabilized in most countries (Parent et al. 2003). However, in the last 15 years there seems to be a trend of further decline in age of puberty in the US and in Europe characterized by early onset of growth of pubic hair (pubarche), early breast development (thelarche) and/or age of menstruation (menarche) (Aksglaede et al.

2009, Euling et al. 2008, Rubin et al. 2009, Mouritsen et al. 2010). This new decline does not seem to be associated with any major change in socio-economic conditions and the advancement in pubertal timing happening rapidly over the past decades indicates an environmental etiology (Sorensen et al. 2012). Some factors that may affect maturation of the reproductive system are changes in frequency of obesity, fetal nutrition, childhood dietary habits, physical activity and also exposure to endocrine disrupting chemicals (Adair 2001, Den Hond et al. 2002, Georgopoulos et al. 1999, Kaplowitz et al. 2001, Mouritsen et al. 2010, Parent et al. 2003).

Supporting the possible contribution of EDC exposure on the changes of timing of sexual maturation are several animal studies (some examples were mentioned in the previous section) (Gray et al. 1989, Kim et al. 2002, Laws et al. 2000a, Ma et al. 2006). There are also examples from human studies for example associations between age of menarche and breast development and serum levels of for example phthalates and DDT (Buck Louis et al. 2008, Colon et al. 2000, Ouyang et al. 2005, Wang et al. 2005) and early menarche and pubic hair development in girls exposed *in utero* and via breastfeeding with high levels of brominated flame retardeants (Blanck et al. 2000). Furthermore, although the majority of chemicals have not been studied in both humans and animals, there are a few examples of chemicals where data seems to point the same way. For example TCDD has been shown to delay puberty in female rats (VO) (Gray and Ostby 1995) and increased serum levels are associated with delayed breast development in humans (Den Hond et al. 2002).

2.4.3 Differences and similarities between rodents and human

The time of onset of puberty is affected by both genetic and environmental factors in the human as well as in the rat (Kaprio et al. 1995, Parent et al. 2003, Rivest 1991). Both humans and rats are regular cycling, display spontaneous ovulation, and have midcycle gonadotropin surges that trigger comparable follicular and oocytic changes in the ovaries (Buck Louis et al. 2008). In rodents GnRH secretion is maintained at low levels during juvenile life by steroid control whereas in humans it is regulated by central mechanisms independent of gonadal regulatory inputs (Urbanski and Ojeda 1987). The time frame of puberty is different between the species (Gray et al. 2004) and so are the puberty landmarks evaluated which in humans are breast and pubic hair development and menarche (Rockett et al. 2004). Still the activation at puberty of GnRH release in rodents and primates are initiated and regulated by similar inhibitory and stimulatory pathways making the rat a suitable and widely used model (Buck Louis et al. 2008).

2.5 Estrous cycle

A major focus in this thesis has been on estrous cycle measurements. Evaluating changes in vaginal epithelial cell structure has been used for decades to document the reproductive cycle in laboratory mice and rats and to contribute to the assessment of the functional status of the hypothalamic-pituitary-ovarian axis in

reproductive toxicology studies (Goldman et al. 2007). In the rat the cyclical changes of the vaginal histology during the estrous cycle was first described by Long and Evans (1922). In rats cycling begins shortly after onset of puberty as marked by vaginal opening. Initially, the rats show some irregular cycles but by around 45 days of age it will begin to show a regular pattern with cycle lengths normally lasting for four or five days (Cooper and Goldman 1999). The estrous cycle can be described by four stages each based on characteristic composition of cell cytology in the vaginal smear.

The four stages of the estrous cycle are called: proestrus (P), estrus (E), metestrus (M) and diestrus (D) and normally occurs in the order P-E-M-D in a four day cycle (Goldman et al. 2007). Because rats cycle continually throughout the year (continuously polyestrous), diestrus is immediately followed by the proestrus stage of the next cycle. Anestrus, which is a period of reproductive rest state between estrous cycles, is not usually observed in healthy, cycling female rats. Normally, estrous cyclicity only stops during pseudopregnancy, pregnancy, and lactation. Figure 7 shows the length of each stage of the estrous cycle.

Environmental factors, e.g. the presence of males, light/ dark cycles have an impact on the regularity of the estrous cycles and should be considered before smears are collected in any experiment. Smears should be collected approximately at the same time every day (OECD 2009c).

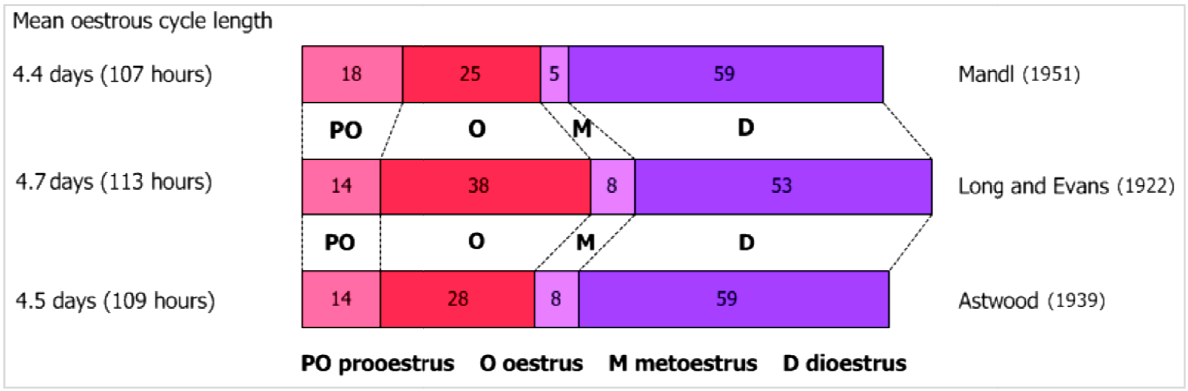


Figure 7. Duration of the four stages of the estrous cycle in the laboratory rat as reported by Long and Evans (1922), Astwood (1939) and Mandl (1951) (Astwood 1939, Long and Evans 1922, Mandl 1951, OECD 2009b).

2.5.1 Hormonal fluctuations and the estrous cycle

The cyclic changes occurring in the vaginal cell morphology are initiated and regulated by the hypothalamic-pituitary-ovarian (HPO) axis. This section will focus on the major reproductive hormones LH, FSH, estradiol, progesterone and inhibin. The estrous cycle is regulated by a complex system of feedback mechanisms between the hypothalamus, the pituitary and the ovaries involving both negative and positive feedback mechanisms of various hormones depending of the stage of estrous cycle which is shown in Figure 8 (OECD 2009b). The relative levels of the hormones FSH, LH, estradiol and progesterone and how they vary in the estrous cycle are shown in Figure 9.

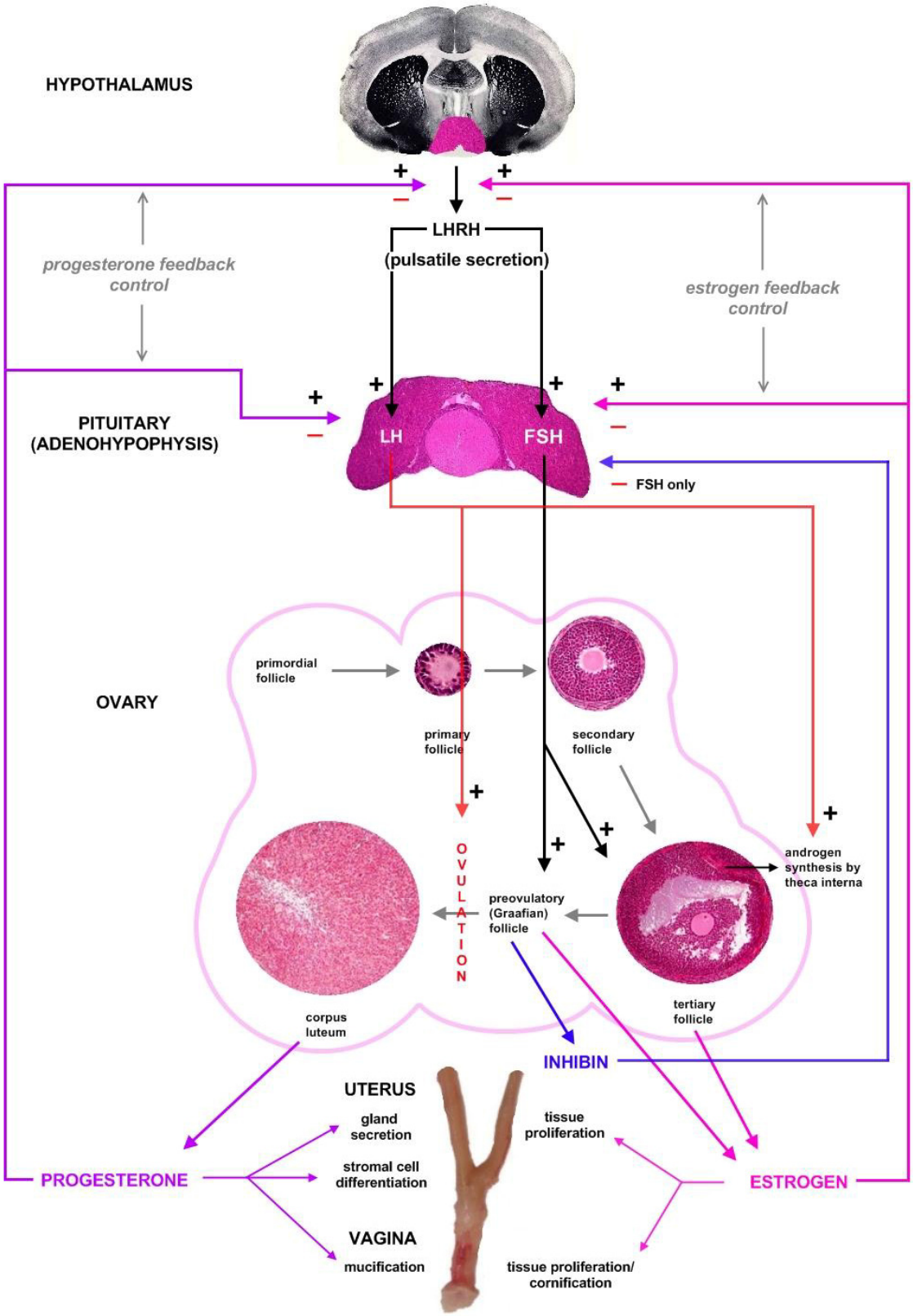


Figure 8. The Hypothalamus-pituitary-ovary axis showing the main interactions between compartments of the axis via the major hormones during the estrous cycle. From (OECD 2009d).

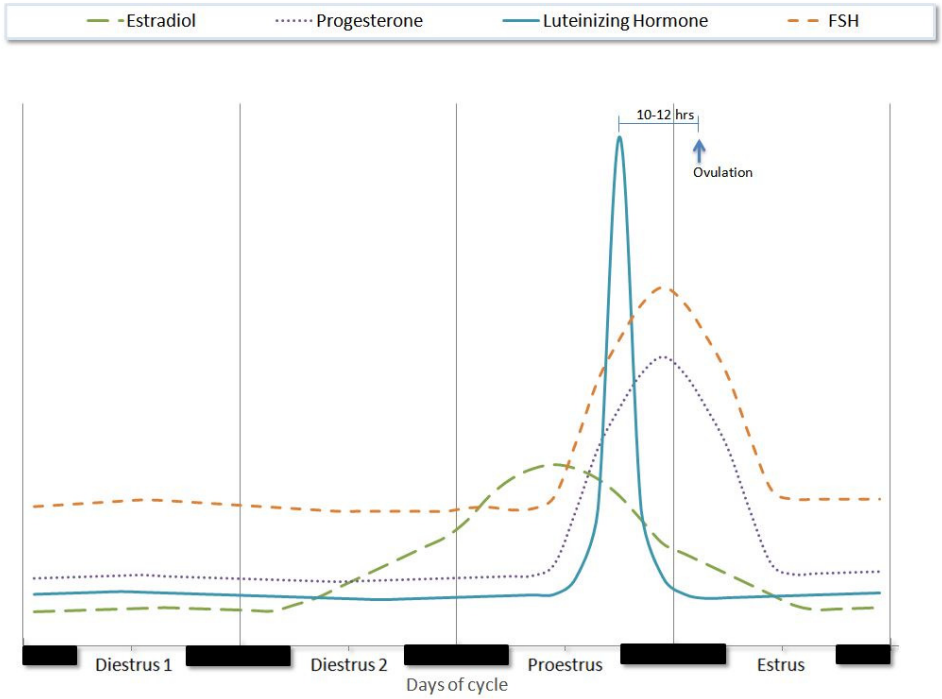


Figure 9 Modified after Goldman et al (2007) and Butcher et al 1974, (Butcher et al. 1974, Goldman et al. 2007). The figure is showing schematically how the hormone levels of LH, estradiol and progesterone are fluctuating during the estrous cycle of the rat. The surge in LH is followed by ovulation 10-12 hours later. In this figure metestrus is called diestrus 1 and diestrus is called diestrus 2.

FSH: In diestrus follicular growth is stimulated by the pituitary gonadotropins (in turn regulated by GnRH from the hypothalamus), primarily follicle stimulating hormone (FSH). FSH rises preovulatory, peaks and returns to base levels halfway through estrus (Butcher et al. 1974).

Estradiol: The follicular growth is accompanied by a rise in secretion of estradiol which peaks halfway through proestrus. At this point of the estrous cycle the level of estradiol is rising and estradiol is suppressing the release of GnRH from the hypothalamus and also exerts a direct effect on the pituitary inhibiting secretion of LH and FSH. Further negative feedback of pituitary FSH secretion is achieved by the production of inhibin in the maturing follicle (Woodruff et al. 1996). When estradiol levels have peaked the inhibitory effect on GnRH and gonadotropin secretion come to an end and estradiol is now exerting a positive feedback on the hypothalamic-pituitary system promoting GnRH release and increasing pituitary responsiveness to GnRH leading to a preovulatory surge in LH (OECD 2009b).

Progesterone: Levels of ovarian progesterone also rise starting at early proestrus and peaks a few hours later than estradiol. Like in the case of estradiol, the feedback of progesterone to the hypothalamic-pituitary system can be positive or negative depending on the stage of the estrous cycle. The rising progesterone levels during proestrus increases hypothalamic release of GnRH and promotes (in concert with estradiol) the

preovulatory LH surge. Post ovulation (ovulation happens early in the estrus stage) progesterone (as with estradiol) inhibits gonadotropin secretion (OECD 2009b).

LH: During the estrous cycle the level of Luteinizing hormone (LH) remains low but a peak in the concentration happens at the day of vaginal proestrus and the surge in LH is followed by ovulation 10-12 hours later (Cooper and Goldman 2010). The LH surge is released from the anterior pituitary in response to GnRH.

The sequential rise of the ovarian hormone estradiol followed by progesterone affects the hypothalamus and pituitary and controls the amount and timing of the LH surge (Cooper and Goldman 2010). In turn the LH surge starts the final stage of oocytic and ovarian follicular maturation that comes before the ovulatory rupture of the follicle (Goldman et al. 2007).

2.5.2 Types of chemicals that can insult the HPO system thereby affecting estrous cyclicity

In principle chemicals that interfere with any target along the HPO- axis including excretion and production of hormones could disrupt the estrous cycle and block ovulation. A disruption of cycling caused by chemical exposure can induce an acyclicity characterized by a persistent estrus, a persistent diestrus, or cause an irregular pattern with cycles of abnormal duration (Goldman et al. 2007). A regular cycle is not; however, necessarily proof that a chemical is not a reproductive toxicant as a cycle can still be anovulatory (Cooper and Goldman 1999). Examples of classes of chemicals that can disrupt normal estrous cyclicity include pesticides as well as chemicals used in production. Some examples of chemicals that disrupt estrous cycle are: atrazine, which probably acts through a hypothalamic mechanism (Cooper et al. 1996, Eldridge et al. 1999), estrogenic (e.g. methoxychlor, nonylphenol, BPA (Okazaki et al. 2001, Woo et al. 2007)), chemicals that can destroy follicles (e.g. 4-vinylcyclohexene (Lohff et al. 2005, Mayer et al. 2002)), androgens (e.g. 17α-methyltestosterone, oxymetholone (Clark et al. 1998, Okazaki et al. 2002)) and aniandrogens (vinclozolin, DEHP (Hirosawa et al. 2006, Schneider et al. 2011)). As many chemicals probably have more than one mode of action it is not always easy to predict how an estrogenic or antiandrogenic chemical affects the cycle specifically. For example have perinatal exposure to BPA been reported to induce extended periods of diestrus in some animals and extended periods of diestrus and/or estrus in other animals (Rubin et al. 2001) while the effects of e.g. estrogenic chemicals can also be different at high and low doses (Goldman et al. 2007).

2.5.3 The relevance of estrous cycle evaluation in toxicology

Commonly used classes of chemicals have both been shown to cause cycle irregularities in rats and also revealed an association to cycle effects in humans in epidemiological studies. For example the polychlorinated biphenyls (PCBs) and dioxins, which are associated with cycle irregularities in both rats and humans (Brezner et al. 1984, Chao et al. 2007, Li et al. 1995, Meerts et al. 2004, Yang et al. 2005). This also

seems to be the case in both rats and humans for some agricultural pesticides, including herbicides and fungicides, like DDT, atrazine, lindane and mancozeb (Baligar and Kaliwal 2001, Chadwick et al. 1988, Cragin et al. 2011, Eldridge et al. 1999, Farr et al. 2004, Gotz et al. 2001, Heinrichs et al. 1971, Lahiri et al. 1985, Windham et al. 2005). Care should of course be taken when interpreting epidemiological data as there are many confounding factors when dealing with a human population.

There are some differences among the estrous cycle of the female rat and women that one should keep in mind: The rat has a 4- to 5-day estrous cycle and corpus luteum is not sustained if mating does not occur. Corpora luteal function is sustained for approximately 10-16 days by mating-induced cervical stimulatory prolactin surges in rats. Humans have a menstrual cycle around 28 days in duration and do not have periods of peak behavioral estrus during the cycle. Human menstrual cycle has a spontaneous luteal phase of 10 to 14 days after ovulation as opposed to the rat. The female rat displays sexual receptivity only during estrus (Gray et al. 2004). Furthermore, other biomarkers are more useful in assessing the human ovarian cycle than vaginal cytology, like blood hormone measurements, menses or alterations in vaginal pH (Bretveld et al. 2006, Goldman et al. 2007).

With these differences between rodents and humans in mind, evaluation of rodent cyclicity can still be of value when considering potential adverse effects in women. Many of the underlying endocrine mechanisms associated with successful follicular development, ovulation, pregnancy, and parturition are homologous between the species (Bretveld et al. 2006, Cooper and Goldman 1999, Goldman et al. 2007, Gray et al. 2004). Also the hormone profile during the estrous and menstruation cycle is similar between rodents and women (Ben-Jonathan et al. 2008). For this reason, a chemical induced inhibition of ovarian cycles in female rats should suggest that a compound may be a reproductive toxicant in women.

2.6 Female reproductive senescence in rats and humans

In this thesis, some emphasis has been placed on effects on the female system which become evident later in life compared to the time of exposure. One such effect is the time of reproductive senescence.

Rodents begin to exhibit irregular cycles at 9–12 months with differences between strains (Maffucci and Gore 2006). When the aging female rat starts getting irregular cycles, it typically displays elongated cycles dispersed between regular cycles and gradually becomes more irregular. The irregularly cycling rats have prolonged cycles of 6 days or more, and acyclic rats are in persistent estrus or persistent diestrus (LeFevre and McClintock 1988, Rubin 2000).

In humans, menopause is defined as a permanent cessation of menstruation and is clinically diagnosed after one year of amenorrhea (absence of a menstrual period) (Kermath and Gore 2012, Maffucci and Gore 2006, World Health Organization (WHO) 1996). The mean age for women to reach menopause is 51 years and at

menopause the ovarian follicular reserve has been exhausted (Wu et al. 2005). The menopause is preceded by a period of irregular menstrual cycles, hormone fluctuations and decreased fertility that normally occurs between ages 45 and 54 and this period is termed perimenopause and lasts till one year after last menstruation (Fitzgerald et al. 1998, Nejat and Chervenak 2010, Santoro et al. 1996, Soules et al. 2001). Timing of reproductive senescence is determined by both genetic and environmental factors (Gore et al. 2011, Kok et al. 2005).

Most research has been considering the genetic factors but there is growing evidence that environmental factors like for example cigarette smoking (Midgette and Baron 1990) and exposure with endocrine disrupters can affect timing of menopause and thereby shorten a person's duration of reproductive life (Gore et al. 2011). In mammals the total ovarian reserve of follicles has been believed to be established before birth or soon thereafter, and anything that could decrease the total follicle pool can theoretically result in early menopause (Diamanti-Kandarakis et al. 2009). There has, however, been some evidence that in juvenile and adult mice a recruitment of ovarian follicles may be possible later in life (Johnson et al. 2004, Johnson et al. 2005a). This matter has been subject to some skepticism (Byskov et al. 2005, Johnson et al. 2005b, Telfer et al. 2005, Tilly and Johnson 2007) and in any event the follicle reserve decreases with age in women with very few remaining at menopause (Wallace and Kelsey 2010).

Earlier it was believed that menopause was just a result of depletion of follicular reserve and that the changes in hypothalamic and pituitary hormones accompanying menopause was a result of ovarian failure (vom Saal et al. 1994). There is, however, evidence from studies on rats that their hypothalamic and pituitary systems also undergo aging (Peng and Huang 1972, Rubin 2000) and there also seems to be an aging of the HPO axis in women (Kermath and Gore 2012, Shaw et al. 2009, Weiss et al. 2004). It has been hypothesized that this may be involved in the accelerated decrease in follicle numbers observed in women the last decade up until menopause (Faddy and Gosden 1996, Richardson et al. 1987, Wise et al. 1997).

Menopause decreases fertility in late reproductive years and since many women choose to delay childbirth because of careers, birth control and other considerations (Fitzgerald et al. 1998, Wu et al. 2005) a chemically induced earlier menopause may give rise to problems conceiving for some women. Furthermore, menopause affects women's risk of getting chronic diseases associated with decreased levels of reproductive hormones (Farr et al. 2006). In fact menopause is associated with increased risk of diseases in the cardiovascular system (Chae and Derby 2011), bone density (Lo et al. 2011) and possibly effects on cognitive performance (Rocca et al. 2011, Sherwin 2006, Zandi et al. 2002).

2.6.1 Differences and similarities between women and rats and relevance of measuring onset of estropause in rodents

Rodents do not have a menstrual cycle and the process of reproductive aging may more correctly be called estropause instead of menopause. Nevertheless, rats undergo reproductive failure in middle age that in many ways resembles what happens in humans in the perimenopausal transition. Some similarities and differences will be considered in the following section.

When reproductive decline is imminent an increase in FSH is observed in both humans and rats at middle age (DePaolo 1987, Klein and Soules 1998, Rubin 2000, Wise et al. 2002). Also Inhibin B levels decrease in both rats and women (Kermath and Gore 2012, Rubin 2000, Sowers et al. 2008).

LH secretion changes in women and rats as they enter the perimenopausal transition and cycle length in both women and in the rat become greatly variable (LeFevre and McClintock 1988, Sherman et al. 1976, Wise et al. 2002). In rats there is an attenuation and delay of the LH surge and a decrease in fertility, similar to humans (Rubin 2000, Van Kempen et al. 2011, Wise et al. 2002). Women in perimenopause transition have estrogen levels comparable or increased compared to young women but the levels fluctuate (Kermath and Gore 2012, Santoro et al. 1996) which is similar to what is observed in rats entering irregular cycles (Wise et al. 2002). Progesterone declines with age in humans as well as in female rats (Klein and Soules 1998, Lu et al. 1979, Santoro et al. 1996) but the levels are not as reduced in rats as in humans (Van Kempen et al. 2011).

A major difference between rat and human menopause is that although a decline in follicular reserve is observed in middle aged female rats they retain a much higher number of follicles resulting in a higher level of estrogens than is the case in menopausal women, who has almost exhausted the ovarian follicle pool at the time of menopause (see Figure 10) (Klein and Soules 1998, Mandl and Shelton 1959, Rubin 2000, Wallace and Kelsey 2010). Aging at the hypothalamus - pituitary level seems to play a very important role in the onset of irregular estrous cycles in the rat (Maffucci and Gore 2006, Peng and Huang 1972, Rubin 2000).

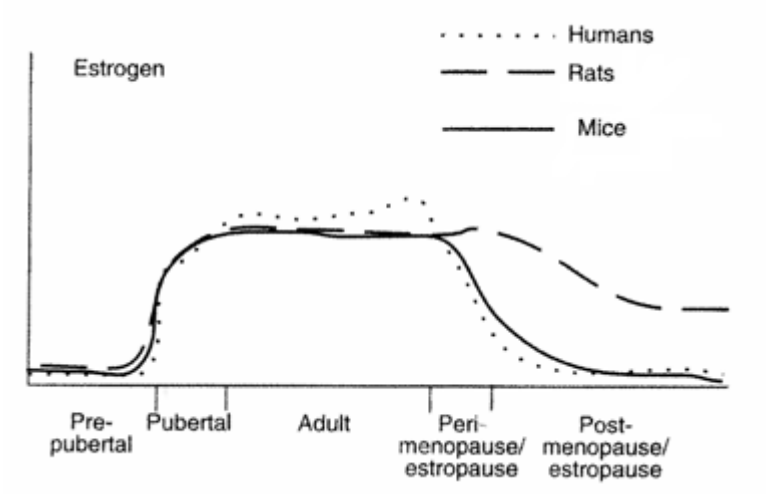


Figure 10. The profile of estrogen levels during the reproductive periods of a lifetime, modified from (Maffucci and Gore 2006). The rat estrogen levels stay higher later in the reproductive aging process than the levels in humans and mice.

Because of the similarities between rats and humans in reproductive aging in the transition state the intact rat is often considered a good model for the perimenopausal woman but due to the difference in estrogen levels and follicle reserve between human and rat later in the process, it is not as good a model for human postmenopause (Kermath and Gore 2012, Rubin 2000, Wise et al. 2002).

Onset of estrous cycle irregularities has been used to measure effects on reproductive senescence for a number of chemicals. Examples of chemicals that induces early onset of irregular estrous cycles in rats are methoxychlor, bisphenol A, and dioxin (Adewale et al. 2009, Armenti et al. 2008, Gore et al. 2011, Shi et al. 2007). Methoxychlor exposure to 100 mg/kg bw/day perinatally, induced a significantly higher proportion of irregularly cycling female rat offspring compared with control at 13 months of age (Gore et al. 2011). In a study on bisphenol A only 33% in the groups of rats neonatally treated with 50 mg/kg bw/day and 86% of the animals treated with 50 µg/kg bw/day had regular estrous cycle at 15 weeks after VO compared to 100% in vehicle control (Adewale et al. 2009). Oral exposure to TCDD GD14, 21 and PND 7 and 14 of female rat dams caused accelerated transition to reproductive senescence, measured as a decrease in the number of regularly cycling female offspring compared with control, in a dose-responsive manner beginning at 9 months of age in groups receiving 50 or 200 ng/kg/week (Shi et al. 2007).

2.7 Anti-Müllerian hormone an endpoint used to assess ovarian reserve.

Anti-Müllerian hormone (AMH) is a member of the growth factor-beta family which acts on tissue growth and differentiation. AMH is also known as Müllerian inhibiting substance (MIS) (La Marca et al. 2010, Yeh et al. 2007). In the fetal testis it is produced by the Sertoli cells and induces regression of the Müllerian ducts (Lee and Donahoe 1998, Visser et al. 2006).

In the ovary, AMH is involved in inhibition of the early stages of follicle growth (Carlsson et al. 2006, Durlinger et al. 2001, Durlinger et al. 2002, Visser et al. 2006) and may regulate the responsiveness of growing follicles to FSH by inhibiting the sensitivity to FSH. AMH is produced primarily by the granulosa cells of preantral/primary and small antral follicles of the ovary and is not expressed in the final FSH-dependent stages of follicle growth (figure 11) (Durlinger et al. 2002, Kelsey et al. 2011, Visser et al. 2006). This is a great advantage of AMH as a biomarker because it means that levels of AMH in plasma are quite stable across cycle and also between cycles in the same individual (Cook et al. 2000, Fanchin et al. 2005, La Marca et al. 2004). It has been shown to decline with age in humans, rats, and mice and in humans it is used as a predictor of fertility/reproductive aging (Kevenaar et al. 2006, Visser et al. 2006, Yeh et al. 2007). The levels of AMH in blood are proportional to the number of developing follicles in the ovaries which is indirectly reflecting the number of primordial follicles, or the ovarian reserve (Broekmans et al. 2008). Since the serum levels of AMH have been shown to correlate with follicle pool also in rats (Yeh et al. 2007) it could have the potential to be used as an endpoint in toxicological tests when assessing exhaustion of follicle reserve due to chemical insult. Indeed, it has been used as a biomarker to assess the degree of ovarian damage caused by exposure to the chemotherapeutic agent cisplatin (Yeh et al. 2006).

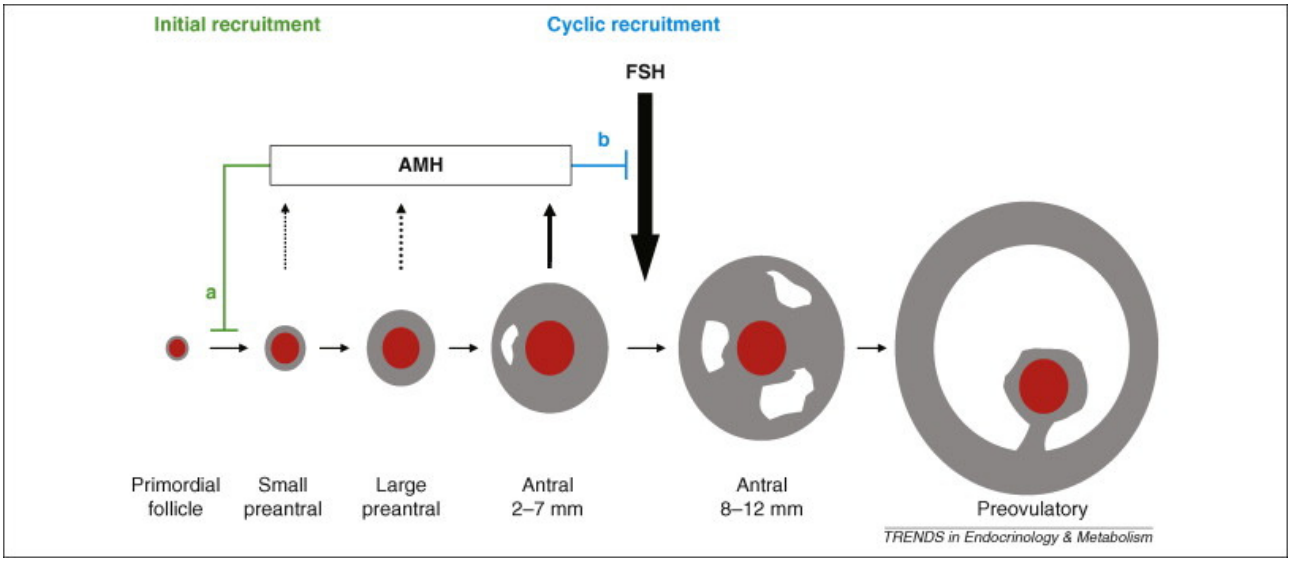


Figure 11. Model of AMH action in the ovary, figure modified from (Broekmans et al. 2008). The known role of AMH in normal ovarian follicle development is shown (the red centre represents the oocyte, the grey area represents the granulosa cell layer and the white area represents follicle fluid in the antrum). AMH is expressed in small and large preantral follicles (broken arrows) and in small antral follicles (unbroken arrow). Initial recruitment is a continuous process, whereas cyclic recruitment is driven by a rise in FSH serum levels at the end of a previous menstrual cycle. Inhibitory effects of AMH on follicle development are on (a) the initial recruitment of primary follicles from the resting primordial follicle pool and (b) on the sensitivity of antral follicles for FSH.

2.8 Mixtures of chemicals

As has been outlined in the previous sections, the endocrine system can be affected by many types of chemical compounds. With a few exceptions, chemical risk assessment considers only the effects of single substances. Toxicity of mixtures are important, as humans are likely to be exposed to several endocrine disruptive compounds simultaneously and from multiple sources like cosmetics, food, water, air and dust, rather than just one chemical at a time. Exposure scenarios are complex, however, mixture effects are necessary to include in risk assessment, since assessment based solely on single compounds can possibly underestimate the risk (Kortenkamp et al. 2010). Indeed, *in vitro* as well as *in vivo* reproductive toxicity studies have shown that certain endocrine disrupting chemicals are able to contribute to the overall effect of a mixture, even when the individual components of the mixture are present at concentrations below the threshold of statistically detectable effects (Christiansen et al. 2008, Christiansen et al. 2009, Hass et al. 2007, Rajapakse et al. 2002, Rider et al. 2008, Silva et al. 2002). Since it is not possible in practice to test all possible combinations of chemicals in mixture studies, models that can predict joint effects of mixtures on the basis of data obtained from testing single chemicals are greatly needed in chemical risk assessment (Cedergreen et al. 2008, Kortenkamp et al. 2010).

The two most commonly used models to predict effects of mixtures are the concept of dose addition (DA) introduced by Loewe and Muischnek (Loewe and Muischnek 1926) and independent action (IA) introduced by Bliss (Bliss 1939). Both models are based on the assumption that there is no interaction (physically, chemically, or biologically) between the compounds in the mixtures, in other words, one compound does not influence the toxicity of the other compounds (Cedergreen et al. 2008).

The IA model assumes that each chemical in a mixture has its own unique mechanism of action (dissimilar acting). The DA model is based on the assumptions that the chemicals in the mixture act on the same site or has the same effects and can be considered as dilutions of one another differing only in their individual potency (similar action). Similar action, however, can be interpreted in a very broad or a very strict sense either comprising all substances capable of cause a particular toxicological response i.e. antiandrogens or only those acting at a particular molecular binding site (Faust et al. 2001).

Recently, the DA model has been shown to be valid to both similar and dissimilar acting compounds *in vivo*. In some cases the DA model has even made predictions more closely in accordance with the observed effect than predictions made using the IA model (Christiansen et al. 2009, Rider et al. 2008).

There is an important difference between the predictions based on IA and DA that can be of importance for risk assessment. If the exposure to chemicals in a mixture is at or below the no observed adverse effect levels (NOAELs), the IA model will predict no adverse effect of exposure to the mixture whereas DA can predict that adverse effects will in fact occur even if all the chemicals in the mixture are below their individual NOAELs (Rider et al. 2008, Silva et al. 2002).

In the studies included in this thesis four out of five are mixture studies, however, the prediction calculations are not within the scope of this thesis. The endpoints mentioned in the previous sections have all been shown to be affected by exposure to endocrine disrupters in female rodents. These endpoints have been measured in the studies as part of this thesis and in the following chapters the methods, results and discussion of the results will be reported. Moreover, the next sections will also cover some method related considerations.

3 Methods

The results, which are presented in this thesis, were obtained in five developmental toxicology studies. Two studies investigated mixtures of endocrine disrupting pesticides (Pestimix), two investigated mixtures of endocrine disrupting chemicals based on human exposures (Contamed), and one study tested a positive control for estrogenic effects, ethinyl estradiol (EE2). The project with the mixture of the five pesticides included two range-finding studies (collectively called Pestimix RF) and a dose response study (Pestimix DR). In the Contamed project, mixtures were modeled based on high end human intakes, and the project involved two developmental mixture studies in rats, called Contamed 1 and 2. Finally, results from a dose response study on the estrogenic drug EE2 were included. In all of these studies, both male and female offspring were investigated for adverse effect of the exposure, however, the endpoints which were focused on in this thesis, were only in females and included gestational length, anogenital distance (AGD), number of nipples, onset of puberty, measurements of Anti-Müllerian hormone (AMH) and estrous cyclicity at several time point during the animals life span.

3.1 Chemicals, doses and composition of the mixtures

The data included in this thesis originates from several different studies. Pestimix RF consisted of two range finding studies (a and b) but will be treated as one study in this thesis. The mixture ratio was based on doses causing no effects on gestation lengths and pup survival for the individual pesticides, i.e. Pestimix-175 (100%). Table 1 shows the composition of the mixture doses tested in the two range finding studies a and b (paper I). Animals were dosed from GD7 to PD16 except in the three highest doses where the animals were sacrificed GD25 due to impaired parturition in several of the dosed dams. The term pup day (PD) is used in this thesis and the expected day of delivery, GD 23, is designated pup day (PD) 1 for the pups. Thereby, the age of the pups is related to the time of conception, but is rather similar to postnatal age.

Table 1. The composition of mixtures in the Pestimix rangefinding (Pestimix RF). The notation a and b denotes the two studies a and b (in mg/kg bw/day).

Pesticide	**Pestimix-43.75 (25%[b])**	**Pestimix-87.5 (50%[b])**	**Pestimix-131.25 (75%[a])**	**Pestimix-175 (100%[a])**	**Pestimix-218.75 (125%[a])**
Epoxiconazole	3.75	7.50	11.25	15.00	18.75
Mancozeb	6.25	12.50	18.75	25.00	31.25
Prochloraz	8.75	17.50	26.25	35.00	43.75
Tebuconazole	12.5	25.00	37.50	50.00	62.50
Procymidone	12.5	25.00	37.50	50.00	62.50
Pestimix, total	**43.75**	**87.5**	**131.25**	**175.00**	**218.75**

The doses tested in the following dose reponse study Pestimix DR study (Table 2) was chosen based on results obtained in the two range finding studies, where higher doses than 43.8 mg/kg bw/day caused effects on pup survival, and is described in detail in paper II and III. Dams were administered GD7-GD21 and from the day after birth to pup day 16.

Table 2. Doses of the pesticides administered individually and in mixture to pregnant rat dam from GD 7 to pup day 16 (in mg/kg bw/day) and number of dosed time-mated rats (dams with viable litters) in each group. Epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu) or procymidone (Procy).

Group	Epoxiconazole	Mancozeb	Prochloraz	Tebuconazole	Procymidone	Pesticide mixture	% of NOAEL for effect on parturition
1: Control	-	-	-	-	-	-	-
2: Pestimix-14.6	1.25	2.08	2.92	4.17	4.17	14.6	8.3
3: Pestimix-29.2	2.5	4.17	5.83	8.33	8.33	29.2	17
4: Pestimix-43.8	3.75	6.25	8.75	12.5	12.5	43.8	25
5: Epoxi-3.75	3.75	-	-	-	-	-	25
6: Epoxi-15	15	-	-	-	-	-	100
7: Manz-6.25	-	6.25	-	-	-	-	25
8: Manz-25	-	25	-	-	-	-	100
9: Prchl-8.75	-	-	8.75	-	-	-	25
10: Prchl-35	-	-	35	-	-	-	100
11: Tebu-12.5	-	-	-	12.5	-	-	25
12: Tebu-50	-	-	-	50	-	-	100
13: Procy-12.5	-	-	-	-	12.5	-	25
14: Procy-50	-	-	-	-	50	-	100

The Contamed project weas based on human high end exposure to mixtures of environmentally relevant endocrine disrupters and consisted of two studies. These studies will be referred to as Contamed 1 and Contamed 2 and the composition of the mixtures and dose groups can be seen in Table 3. The reasons for choice of chemicals tested the mixtures in the Contamed studies, the dosing scheme as well as reasons for the doses chosen in Contamed 1 are described and explained in paper IV (Christiansen et al. 2012). In Contamed 2 the doses chosen were based on the results obtained in Contamed 1and the Amix (only included antiandrogenic chemicals marked with blue color in table 3), unlike in Contamed 1, did not contain

paracetamol (PM) because it was important to investigate if paracetamol by itself caused the effects observed primarily on the male offspring. Emix only included the chemicals with estrogenic properties (red color in Table 3). During gestation dosing occurred GD7-21 and during lactation the exposure period was day after birth to PD22. Paracetamol was only present in the mixture or in the vehicle treated with 360 mg/kg bw/day paracetamol GD13-19 to avoid effects on implantation and possible effects on birth, respectively (Christiansen et al. 2012, Gupta et al. 1981). Paracetamol was not administered PD1-13 in lactation in order to be able to compare with data from Contamed 1 and data from an earlier study (Kristensen et al. 2011a) and administered PD14-PD22 only to be able to measure blood levels.

Table 3. The composition of the mixtures in mg/kg bw used in Contamed 1 and 2. The total doses are summed up in the bottom of the table. Chemicals in cells with blue coloration were considered to have antiandrogenic modes of action and chemicals in cells with red coloration had estrogenic modes of action. Ratio is the estimated high end human exposure.

Study	**Contamed 1**				**Contamed 2**							
Chemical	**Ratio**	**Mix-150**	**Mix-450**	**Amix-450**	**Mix-100**	**Mix-200**	**Mix-450**	**Amix-200**	**Amix-450**	**Emix-200**	**Emix-450**	**Paracetamol**
DBP	0.01	1.5	4.5	4.5	1	2	4.5	2	4.5	0	0	0
DEHP	0.02	3	9	9	2	4	9	4	9	0	0	0
Vinclozolin	0.009	1.35	4.05	4.05	0.9	1.8	4.05	1.8	4.05	0	0	0
Prochloraz	0.014	2.1	6.3	6.3	1.4	2.8	6.3	2.8	6.3	0	0	0
Procymidone	0.015	2.25	6.75	6.75	1.5	3	6.75	3	6.75	0	0	0
Linuron	0.0006	0.09	0.27	0.27	0.06	0.12	0.27	0.12	0.27	0	0	0
Epoxiconazole	0.01	1.5	4.5	4.5	1	2	4.5	2	4.5	0	0	0
4-MBC	0.06	9	27	0	6	12	27	0	0	12	27	0
OMC	0.12	18	54	0	12	24	54	0	0	24	54	0
ppDDE	0.001	0.15	0.45	0.45	0.1	0.2	0.45	0.2	0.45	0	0	0
Bisphenol A	0.002	0.225	0.675	0	0.15	0.3	0.675	0	0	0.3	0.675	0
Butyl-paraben	0.06	9	27	0	6	12	27	0	0	12	27	0
Paracetamol	0.8	120	360	360	80	160	360	0	0	0	0	360
Sum	**1.12**	**168.17**	**504.50**	**395.82**	**112.11**	**224.22**	**504.50**	**15.92**	**35.82**	**48.30**	**108.68**	**360**

4-MBC=4-methyl-benzylidene camphor, p,pDDE=1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene, OMC= Octyl methoxycinnamate

In the EE2 study, which was originally designed as a positive control study to investigate effects on mammary gland but other endpoints were recorded as well and included in this thesis (Paper V). Four groups of rats were dosed with the vehicle corn oil (control), 5 µg EE2/kg bw/day, 15 µg EE2/kg bw/day or µg EE2/kg bw/day from GD7 to PD21. Husbandry and procedures of sacrifice in this study was as is described in papers I-V. Table 4 provides an overview of the studies in this thesis, the exposure and the number of dams in the groups.

Table 4. The table gives an overview of the studies and the exposure groups providing the female offspring included in this thesis

Study overview		
Group-dose /studies	**Exposure**	**No of mated female rats (pregnant)**
Pestimix RF		
1.Control	Vehicle (corn oil)	16 (8)
2. Pestimix-43.75	25% of NOAEL for effect on parturition and pup survival	8 (4)
3. Pestimix-87.5	50% of NOAEL for effect on parturition and pup survival	8 (8)
4. Pestimix-131.25	75% of NOAEL for effect on parturition and pup survival	8 (7)
5. Pestimix-175	100% of NOAEL for effect on parturition and pup survival	16 (14)
Pestimix DR		
1.Control	Vehicle (corn oil)	22 (15)
2. Pestimix-14.58	8.3% of NOAEL for effect on parturition and pup survival	22 (17)
3. Pestimix-29.17	17% of NOAEL for effect on parturition and pup survival	22 (10)*
4. Pestimix-43.75	25% of NOAEL for effect on parturition and pup survival	22 (15)*
5. Epoxiconazol-3.75	Epoxiconazole (equal to the amount present in the high mixture group 4)	12 (8)
6. Epoxiconazol-15	Epoxiconazole	10 (4)
7. Mancozeb-6.25	Mancozeb (equal to the amount present in the high mixture group 4)	12 (5)
8. Mancozeb-25	Mancozeb	10 (7)
9. Prochloraz-8.75	Prochloraz (equal to the amount present in the high mixture group 4)	12 (8)
10. Prochloraz-35	Prochloraz	10 (4)
11. Tebuconazol-12.5	Tebuconazole (equal to the amount present in the high mixture group 4)	12 (8)
12. Tebuconazol-50	Tebuconazole	10 (6)
13. Procymidon-12.5	Procymidone (equal to the amount present in the high mixture group 4)	12 (7)
14. Procymidon-50	Procymidone	10 (4)
Contamed 1		
1.Control	Vehicle (corn oil)	14
2. Mix-150	All 13 chemical substances (Amix+Emix+PM)	14
3. Mix-450	All 13 chemical substances (Amix+Emix+PM)	14
4. Amix-450	Env. Anti-androgens and paracetamol	14
C ontamed 2		
1.Control	Vehicle	20 (20)
2. Mix-100	All 13 chemical substances (Amix+Emix+PM)	20 (19)
3. Mix-200	All 13 chemical substances (Amix+Emix+PM)	16 (14)
4. Mix-450	All 13 chemical substances (Amix+Emix+PM)	18 (16)
5. Amix-200	Antiandrogens	16 (15)
6. Amix-450	Antiandrogens	16 (16)
7. Emix-200	Estrogens	16 (16)
8. Emix-450	Estrogens	18 (17)
9. Paracetamol-360	Paracetamol	16 (16)
EE2		
1. Control	Vehicle (corn oil)	10 (8)
2. 5	5 µg EE2/kg bw/day	10 (6)
3. 15	15 µg EE2/kg bw/day	10 (9)
4. 50	50 µg EE2/kg bw/day	10 (8)

(Denotes number of pregnant female rats) * In group 3 one dam had only still born pups or killed the rest if any alive and in group 4, one female had to be euthanized due to dystochia. The doses are in mg/kg /bw/day except in the EE2 study where the doses are shown in µg/kg bw/day. The number following the name of the chemical or mixture refers to the dose in mg/kg bw/day.

3.2 Gestational length

From day 21 of gestation the animals were checked twice a day for signs of parturition. The animals were checked for signs of discomfort and bleeding from the vaginal area. Parturition was considered to be completed when the dam had cleaned herself, the pups and the cage for traces of blood and the pups were placed in the nest. The level of precision was 0.5 day, i.e. animals that have given birth in the morning at 8 am of e.g. GD23 was recorded as 23. If they gave birth during GD23 but before 4 pm on GD23 they were recorded as GD23.5.

3.3 AGD, number of nipples and onset of puberty:

After delivery, the weights of dams and individual pups were recorded and the pups were counted, sexed, checked for anomalies and anogenital distance (AGD) was measured using a stereomicroscope. Additionally, anogenital index (AGDI), i.e. AGD/cubic root of body weight, was calculated for all offspring. Pups found dead were macroscopically investigated for changes when possible. On PD 13 (PD14 in the EE2 study because the pups seemed small and areolas/nipples were difficult to see), all female offspring were examined for the presence of areolas/nipples described as a dark focal area (with or without a nipple bud).

Onset of puberty was registered daily in all weaned female offspring until positive. In female offspring sexual maturity was assessed by determining day of vaginal opening (VO) as described by (Goldman et al. 2000). Registrations were performed in females from PD 30 until all had developed VO in Pestimix DR and since in this study some of the animals already displayed VO on day 30 (and registered as having attained VO PD 30, in the following studies the registration began a few days earlier, namely PD 27 (Contamed 1&2 and EE2 studies) until VO was detected in all animals. The animals that already had developed VO on the first day of registration were registered as having VO that first day in all the studies. Age and body weight were recorded on the day that vaginal opening was first observed.

3.4 Estrous cycle

3.4.1 Vaginal Smear

Vaginal smears were collected every day for 15 or 21 consecutive days in the studies included in this thesis (Table 5). Estrous cycle measurements were not performed in Pestimix RF study as the study stopped when the offspring were 3 weeks old. In Pestimix DR and Contamed 1 smears were collected for 15 days starting when the female rats were 5-6 months old and 5 months, respectively. To evaluate potential effect on menopause, smears were also obtained at two later ages in Contamed 1 that is at 9-10 months and again in about 12 months old rats. In Contamed 2 the collection of smears were at 3 months of age and in 12 months old rats. Smears were collected at 2.5 monts of age in the EE2 study. In the two last periods of collecting smears in Contamed 1 and in both studies Contamed 2 and EE2, vaginal smears were collected for 21 consecutive days to acquire better data. An overview is provided in Table 5.

Table 5. Number of periods of collecting smears in the studies and the age of the rats at the time.

	Number of periods collecting smear	**Age at smear (number of days)**
Pestimix DR	1	5-6 months (15)
Contamed 1	3	5 months (15), 9-10 (21) months, 12 months (21)
Contamed 2	2	3 months (21), 12 months (21)
Ethinyl Estradiol (EE2)	1	2.5 months (21)

Vaginal smears were collected at approximately the same time every day between 8 and 10 am in the beginning of the dark period for the animals. A swab moistened in saline was inserted in to the vaginal lumen and cells were transferred to a glass microscope slide. The samples were then allowed to air dry. When dry the smears were fixed in 96% ethanol and stained with Gill's hematoxylin, Orange G6 and eosin-azure 50 (provided by VWR, Gentofte, Denmark) according to the adapted Papanicolaou (PAP stain) procedure reported by (Hubscher et al. 2005). After staining the smears were mounted in Eukit. The stained smears were examined blindly to exposure group by light microscopy and stages were classified as Estrus (E), Metestrus (M), Diestrus (D) or Proestrus (P) or transitions between stages. These stages were recognized by the presence, absence or proportional numbers of epithelial cells, cornified cells and leucocytes as described in OECD guidance document 106 and by Goldman and coworkers (Goldman et al. 2007, OECD 2009a).

Evaluating the results the animals were categorized as either being regularly cycling (cycles lasting 4 to 5 days), or as being irregularly cycling which was defined as having cycles lasting less than 4 days or more than 5 days (e.g. 3 or 7 days) (Cooper and Goldman 1999). Furthermore, episodes of 3-4 consecutive days of vaginal estrus and 4-5 days of diestrus were considered extended. Cycles of longer duration were considered abnormal. Pseudo pregnancy was recognized as a period of 12 – 16 consecutive days of diestrus often characterized by the presence of strings of mucus in the smear and pyknotic cells (except in the 9-10 months old and one-year-old animals because long cycles could be a sign of beginning menopause) (Goldman et al. 2007).

In pestimix DR and in Contamed 1 in the five months old rats, every day before smears were collected, a rat Vaginal Impedance Checker (VIC), Model MK-10C (Muromachi, Japan) was used. The VIC measures the electrical impedance of the epithelial cell layer of vaginal mucosa by inserting a probe into the vagina. If the measured value is 3 kohm (kΩ,) of impedance or above, the animal is considered to be in proestrous. Since the female rat proestrous lasts for approximately 12-18 hours (Hartman 1944, Mandl 1951) it might be possible to use the VIC to assess cycle length. If possible this would be an advantage because the smearing procedure is both very time consuming and demands relatively well trained personnel reading the smears.

Also, the VIC method may be gentler to the animals than using a swab every day for 2-3 weeks which may irritate the lining of the vagina.

In Pestimix DR an error in the procedure occurred and swabbing was performed before the vaginal impedance measurement leading to possible inaccurate measurements of estrous stage. Unfortunately, the error was discovered too late to retrieve useful data on estrus cycle in this study obtained with the VIC.

3.4.2 Vaginal Impedance Checker vs. smear Contamed 1:

The cyclicity data obtained from VIC were compared to the data obtained from assessing the smears in Contamed 1, 5 months old animals. This comparison was based on the assumption that the smears were correctly scored i.e. the smears were chosen as the "golden standard". However, if the VIC showed proestrus a day before estrous appeared in the smear (even if the smear showed diestrus instead of proestrus on that day), the result from VIC was considered correct because the diestrus might have been an early proestrus not recognized when scoring the smear. Sensitivity and specificity were then calculated. According to Altman 1991 (Altman 1991) **sensitivity** is defined as the proportion of positives (here irregularly cycling animals) that are correctly identified by the test. **Specificity** is defined as the proportion of negatives (regularly cycling) that are correctly identified by the test. Sensitivity is calculated using the following equation (Rockette 2011);

$$Sensitivity = \frac{number\ of\ true\ positives}{number\ of\ false\ negatives\ +\ number\ of\ true\ positives}$$

Whereas, specificity was calculated using the equation;

$$Specificity = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ positives}$$

The definitions were as follows:

- True positives; are the number of times the VIC correctly identifies an irregularly cycling animal as observed in the smear.
- False positives; number of times the VIC incorrectly identifies an animal as being irregularly cycling.
- True negatives; number of times an animal correctly is identified as cycling regularly by the VIC.
- False negatives; number of times an animal incorrectly is identified as cycling regularly by the VIC.

3.5 Methods AMH

AMH was measured in 90 day old animals in the EE2 study, in 11 and 14 months old animals in Contamed 1 and in 3 and 12 months old rats in Contamed 2. On the day blood was drawn the animals were anesthesized with Hypnorm® (fentanyl citrate/flunisone)/ Dormicum® (midazolam) and blood was drawn from the tail vein. Blood was allowed to stand for about 1 hour after which it was centrifuged for 10 min at 3500 rpm. Plasma was then transferred to a vial and stored at -20 °C.

The concentration of AMH was measured in plasma using an Enzyme-linked Immunosorbant Assay (ELISA) kit (CSB-E11162r, Rat Müllerian Inhibiting Substance/Anti-Müllerian hormone, MIS/AMH ELISA Kit, CUSABIO BIOTECH CO., Ltd.). The assay was carried out according to manufacturer's protocol.

The data were generally normalized to an internal standard, which was run in all plates. However, this was not the case for the data from the 10 months old rats in Contamed 1 and 4 months old females in the Contamed 2 study. In the Contamed 2 study the data from the 4 months old rats were furthermore not spread out on all the plates used, but all the controls, 14/15 in Mix-450 and 11/14 in PM were measured on the same plate. The rest of the samples were distributed on several plates.

3.6 Power calculations

Power calculations on data from estrous cycle and AMH measurements were made using the program G*Power (Faul et al. 2009). The linear regression option with assumed binominal distribution and Lyles' procedure and two tails was used in calculations on estrous cycle data. The program was used to estimate the smallest effects possible to detect with the actual distribution of regular and irregularly cycling animals in the control groups in the various studies in this thesis. Furthermore, the number of animals necessary in each group was estimated if a 20% or 50% change in the number of regularly cycling animals was to be detected. A power of 0.8 and α=0.05 was used to perform these calculations. Power calculations were made for data on estrous cycle in all the studies except for Pestimix DR and Contamed 2, 3 months old animals, as there were possible problems with the methods used in these studies so power calculations were not meaningful. Power calculations were based on the control group and the group with the largest effect.

A crude estimate of power calculations, group sizes and size of effects possible to detect with AMH data from Contamed 2, 10 month old animals was also done. These data were selected for the calculations because they could be normalized to an intern standard and this was the study with the highest number of animals. The calculations were based on a simple t-test and did not take into account that we had more than two groups but they were done on litter means. The mean of the standard deviation of the control group and the group with the largest difference in response compared to controls were used in the calculations (the group treated with paracetamol). Since there is almost never equal sample sizes in these studies, the calculations were based on the ratio of number of litters in control and the selected exposed group. The

change in AMH that was possible to detect with a power of 0.8 and α=0.05 was calculated and the number of animals required to detect changes in AMH of 10%, 20% and 50% were found using the ratio of animals in control group to exposed group that was present in that study (Contamed 2).

3.7 Statistics:

For all analyses, the alpha level was set at 0.05. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA, or analysis were done on litter means. Body weight was included as a covariate in analyses when relevant, e.g. for onset of puberty and AGD. A Dunnett post hoc test was used to correct for multiple comparisons. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall-Wallis test was used, followed by a Wilcoxon test for pair wise comparisons. When analyzing concentrations of AMH the microtiter plate was used as a random factor where no standard were available to normalize data among plates. The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and θmax, with θmax being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θmax was decided by considering the global fit (information criterion of Schwarz). Litter effects on number of nipples and over-dispersion in the data were accounted by using Generalized Estimating Equations. Statistical significance were assessed using multiple contrast tests (Dunnett contrasts, global error rate α = 5%, two-sided) (Bretz et al. 2005). These tests were chosen as they are already implemented in the SAS procedure PROC GENMOD which was used for all statistical analysis. Estrous cyclicity data were tested using logistic regression and tested for over dispersion with Deviance and Pearson Goodness-of-Fit tests and correction for over dispersion due to litter effects were used when appropriate. Furthermore, the data was analyzed using the Cochran-Armitage trend test. All analyses were performed using SAS Enterprise Guide 4.3, SAS Institute Inc, Cary, NC, 274 USA.

4 Results

This thesis deals with endpoints related to female reproductive health after exposure to mixtures of chemicals or the positive control EE2 during development. The majority of the results are published in papers I to IV, however, not all the endpoints included in this thesis have been published. The results from the EE2 study are included as a manuscript (submitted) (Paper V). Furthermore, all results from the Contamed 2 remains yet to be published and an overview of all endpoints, both published and unpublished, is provided in Table 6. Under the table is an overview of the endpoints that are published, the relevant study and what paper they were published in.

Effects of exposure were observed on all endpoints in the females except for sexual maturation. Gestational length was affected in the Pestimix RF and DR studies as well as in the EE2 study. AGD was affected both in Pestimix RF and DR studies as well as in the EE2 study. Estrous cycle was affected in the 12 months old animals in Contamed 1 and 2 but no effects were seen when measured at an earlier age either in the Pestimix DR study, the two Contamed studies or the EE2 study. A decrease in the AMH level was measured in some of the groups in the four months old animals in Contamed 2, but no other effects were observed on the level of AMH in plasma. All endpoints will be presented in more detail in the following sections including both published and unpublished data.

Table 6. Overview of effects on the endpoints included in the thesis. NA denotes not applicable.

Study no.	Gestational length	AGD/AGDI Pup Day 1	Nipples	Sexual maturation	Estrous cycle	AMH
1 Pestimix RF (Paper I)	Prolonged gestation length in all doses tested, e.g. from Persimix- 43.75 mg/kg bw/day	Longer AGD and AGDI in females in both Mix-43.75 mg/kg bw/day (25%) and Mix-87.5 mg/kg bw/day (50%)	No effects	NA	NA	NA
2 Pestimix DR (Paper II and III)	Prolonged gestation length at Pestimix-29.17 mg/kg bw/day and 43.75 mg/kg bw/day and at 15 mg/kg bw/day epoxiconazole	Longer AGDI in Pestimix-29.17 mg/kg bw/day (16.67%) and longer AGD and AGDI at Pestimix-43.75 mg/kg bw/day and low dose of epoxiconazole (3.75mg/kg bw/day)	No effects	No effects	No effects in 5-6 months old rats	NA
3 Contamed 1 (Paper IV)	No effects	No effects	No effects	No effects	No effects at 5 months and 9-10 months. Effect at 12 months of age (borderline p-value; p=0.07) in the mixture groups. In group dosed with Mix-450, p=0.02 if Contamed 1 and 2 data from control and Mix-450 groups are analyzed together (12 months old)	No effects seen in 11 and 14 months old animals
4 Contamed 2 (not published)	No effects	No effects	No effects	No effects	No effects at 3 months. There seemed to be an effect in the mixture groups (Mix-100, Mix-200 and Mix-450)) at 12 months of age (not significant). Mix-200 compared with control (p=0.053). In group dosed with Mix-450, p=0.02 if Contamed 1 and 2 data from control and Mix-450 groups are analyzed together (12 months old)	Decreased level of AMH in group 4 and (Mix-450, p<0.05) 9 (PM, p=0.02) in 4 months old animals. No effects in 10 months old females.
5 EE2 (Paper V, in preparation)	Prolonged gestation at 50 µg/kg bw/day	Longer AGD and AGDI at 50 µg/kg bw/day	Increased number of nipples at50 µg/kg bw/day bw(P<0.01)	No effects	No effects in 2.5 months old rats	No effects in 90 days old animals

Paper I. Jacobsen PR, Christiansen S, Boberg J, Nellemann C, Hass U. Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats. Int J Androl. 2010 Apr;33(2):434-42. Endpoints included in this thesis: **gestational length, AGD** (Pestimix RF).

Paper II. Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A, Clemmensen LH, Axelstad M.. Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod Toxicol. 2012 Sep;34(2):261-74. Epub 2012 May 29. Endpoints included: **Gestational length, AGD, Nipples** (Pestimix DR).

Paper III, Jacobsen PR, Axelstad M, Boberg J, Isling LK, Christiansen S, Mandrup KR, Berthelsen LO, Vinggaard AM, Hass U. Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod Toxicol. 2012 Sep;34(2):237-50. Epub 2012 Jun 4. Endpoints included: **Puberty onset** (Pestimix DR).

Paper IV. Christiansen S, Kortenkamp A, Axelstad M, Boberg J, Scholze M, Jacobsen PR, Faust M, Lichtensteiger W, Schlumpf M, Burdorf A, Hass U. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. Int J Androl. 2012 Jun;35(3):303-16. doi: 10.1111/j.1365-2605.2011.01242.x. Epub 2012 Feb 28. Endpoints included: **Gestational length, AGD, nipples** (Contamed 1).

Paper V. Mandrup KR, Jacobsen PR, Isling LK, Axelstad M, Dreisig K, Hadrup N, Vinggaard AM, Hass U, Boberg J. Effects of perinatal ethinyl estradiol exposure in Wistar rats. (submitted to Reproductive Toxicology). Endpoints included: **Gestational length, AGD, number of nipples, onset of puberty, estrous cycle** (EE2 study).

4.1 Gestational length

As can be seen in Table 7, gestational length was prolonged in all exposed groups of pestimix RF and in Pestimix DR the gestational length was longer in the groups receiving Pestimix-29.17 and Pestimix-43.75, but also in the group dosed with 15 mg/kg bw/day epoxiconazole (group 6). In the Contamed studies 1 and 2 there were no significant effects of exposure on gestational length. A significantly prolonged gestation length was observed in the group exposed to 50µg/kg bw/day EE2 ($p<0.001$) (Table 7).

4.2 Anogenital distance

AGD and AGDI are shown in Table 7 with the significant effects shown in bold. Female AGD was consistently and significantly increased in both the Pestimix RF and Pestimix DR studies in the groups dosed with Pestimix-43.75 mg/kg bw/day and above. In Pestimix RF both AGD and AGDI was significantly increased in the group dosed with Pestimix-43.75 mg/kg bw/day and Pestimix-87 mg/kg bw/day.

In Pestimix DR a dose-dependent increase in anogenital distance was seen, with significantly longer AGDI, in the two highest mixture groups compared to controls ($p<0.05$ and $p< 0.001$, respectively). A similar effect was seen in animals exposed to both doses of prochloraz ($p< 0.05$ and $p < 0.01$) and tebuconazole ($p< 0.05$ and $p = 0.001$) and in the group exposed to the low dose of epoxiconazol ($p< 0.01$) but not in female pups exposed to a four times higher dose of epoxiconazole. When data was analyzed as AGD with bodyweight as covariate, besides the significant effect in the group receiving the highest mixture dose, significant effects were only detected in the high doses of mancozeb and prochloraz and the low dose of epoxyconazole.

No effects were observed in Contamed 1 or Contamed 2 compared with control. Significantly increased AGD and AGDI were observed in the group dosed with 50 µg/kg bw/day EE2 compared with the control females ($p<0.01$).

Table 7. Gestational length, anogenital distance and the anogenital distance (AGD) index (AGDI) shown as litter mean ± SEM.

Pestimix RF	**Gestation length**	**AGD PD1**	**AGDI PD1**	**No of mated rats (no. of pregnant dams)**
1.Control	23.0 ± 0.0	13.4 ± 0.2	7.3 ± 0.1	16 (8)
2. Pestimix-43.75	**23.5 ± 0.2***	**15.0 ± 0.04 ***	**8.2 ± 0.1*****	8 (4)
3. Pestimix-87.5	**24.1 ± 0.1****	**14.5 ± 0.2***	**8.2 ± 0.1*****	8 (8)
4. Pestimix-131.25	**24.8 ± 0.1****	-	-	8 (7)
5. Pestimix-175	**24.6 ± 0.1****	-	-	16 (14)
Pestimix DR				
1: Control	23.0 ± 0.0	13.6 ± 0.2	7.4 ± 0.1	22 (15)
2: Pestimix-14.58	23.0 ± 0.0	13.7 ± 0.2	7.6 ± 0.1	22 (17)
3: Pestimix-29.17	**23.5 ± 0.2****	14.1 ± 0.2	**7.8 ± 0.1***	22 (9)
4: Pestimix-43.75	**23.6 ± 0.1*****	**15.0 ± 0.2*****	**8.2 ± 0.1*****	22 (14)
5: Epoxiconazol-3.75	23.1 ± 0.1	**14.9 ± 0.4****	**8.1 ± 0.2***	12 (8)
6: Epoxiconazol-15	**23.8 ± 0.3*****	14.3 ± 0.5	7.8 ± 0.4	10 (4)
7: Mancozeb-6.25	23.0 ± 0.0	14.2 ± 0.4	7.8 ± 0.2	12 (5)
8: Mancozeb-25	23.0 ± 0.0	13.5 ± 0.2	7.5 ± 0.1	10 (7)
9: Prochloraz-8.75	23.2 ± 0.1	14.1 ± 0.2	**7.8 ± 0.2***	12 (8)
10: Prochloraz-35	22.8 ± 0.3	**14.5 ± 0.2***	**8.0 ± 0.1****	10 (4)
11: Tebuconazol-12.5	23.0 ± 0.0	14.4 ± 0.4	**7.9 ± 0.2***	12 (8)
12: Tebuconazol-50	23.1 ± 0.1	**14.5 ± 0.2***	**8.0 ± 0.1****	10 (6)
13: Procymidon-12.5	23.0 ± 0.0	13.4 ± 0.2	7.3 ± 0.1	12 (7)
14: Procymidon-50	23.0 ± 0.0	13.3 ± 0.1	7.3 ± 0.1	10 (4)
Contamed 1				
1.Control	23.0 ± 0.04	10.3 ± 0.1	5.7 ± 0.1	14
2. Mix-150	23.0 ± 0.0	10.4 ± 0.1	5.8 ± 0.1	14
3. Mix-450	23.1 ± 0.1	10.7 ± 0.2	5.9 ± 0.1	14
4. Amix-450	23.0 ± 0.0	10.5 ± 0.1	5.8 ± 0.1	14
Contamed 2				
1.Control	23.1 ± 0.1	10.9 ± 0.1	6.0 ± 0.1	20 (20)
2. Mix-100	23.1 ± 0.1	11.0 ± 0.2	6.0 ± 0.1	20 (19)
3. Mix-200	23.1 ± 0.1	10.4 ± 0.2	5.7 ± 0.1	16 (14)
4. Mix-450	23.2 ± 0.1	11.0 ± 0.2	6.0 ± 0.1	18 (16)
5. Amix-200	23.1 ± 0.1	11.2 ± 0.2	6.1 ± 0.1	16 (15)
6. Amix-450	23.2 ± 0.1	11.1 ± 0.2	6.1 ± 0.1	16 (16)
7. Emix-200	23.0 ± 0.0	10.9 ± 0.1	6.0 ± 0.1	16 (16)
8. Emix-450	23.1 ± 0.1	11.0 ± 0.2	6.1 ± 0.1	18 (17)
9. Paracetamol-360	23.1 ± 0.1	10.7 ± 0.2	5.8 ± 0.1	16 (16)
EE2				
1. Control	23.0 ± 0.0	10.9 ± 0.3	6.1 ± 0.1	10 (8)
2. 5 µg/kg bw/day	23.2 ± 0.2	11.0 ± 0.2	6.1 ± 0.1	10 (6)
3. 15 µg/kg bw/day	23.0 ± 0.0	11.1 ± 0.1	6.3 ± 0.1	10 (9)
4. 50 µg/kg bw/day	**23.7 ± 0.2*****	**11.9 ± 0.3****	**6.7 ± 0.1****	10 (8)

- No data, significant effects are shown in bold. *p<0.05, **p<0.01,***p<0.001

4.3 Puberty onset and nipple retention

Table 8 gives an overview of the results of the registration of number of nipples on PD 13 or 14 and day of VO as well as body weight on the day of VO in female offspring in all the studies where these endpoints were recorded. No effects of chemical exposure were observed on onset of puberty compared to control in any of the studies included in this thesis. Compared to vehicle control the only significant result was the increased number of nipples seen in the group dosed with 50 µg EE2/kg/day ($p<0.01$) (Figure 12). In all the studies some animals had already developed VO on the first day registration was performed and those animals were registered as having attained VO on that day. These animals were found both in control and dosed groups.

Table 8. Number of nipples pup day 13 or 14 (EE2) and mean age of vaginal opening (VO) and body weight at VO ± SEM.

Study	No of nipples	No of litters (nipples)	No litters (VO)	Age at VO (days)	body weight at VO
Pestimix RF					
1. Control	12.2 ± 0.1	4	NA	NA	NA
2. Pestimix-43.75	12.6 ± 0.2	4	NA	NA	NA
3. Pestimix-87.5	12.6 ± 0.2	8	NA	NA	NA
Pestimix DR					
1. Control	12.0 ± 0.1	15	15(#3/2)	34.0 ± 0.4	90.2 ± 3.0
2. Pestimix-14.58	12.5 ± 0.9	17	16(#2/2)	34.7 ± 0.5	86.9 ± 2.1
3. Pestimix-29.17	12.4 ± 0.1	9	8	34.4 ± 0.3	84.7 ± 2.6
4. Pestimix-43.75	12.5 ± 0.1	13	13(#2/2)	33.6 ± 0.5	83.8 ± 1.5
5. Epoxyconazol-3.75	12.4 ± 0.1	8	8(#2/2)	34.4 ± 0.5	92.0 ± 3.8
6. Epoxyconazol-15	12.8 ± 0.2	4	4(#1/1)	34.3 ± 1.4	92.7 ± 5.5
7. Mancozeb-6.25	12.2 ± 0.03	5	5	35.2 ± 0.6	89.4 ± 2.6
8. Mancozeb-25	12.4 ± 0.05	7	7(#1/1)	34.0 ± 0.3	85.4 ± 1.4
9. Prochloraz-8.75	12.3 ± 0.1	8	8	34.1 ± 0.5	85.2 ± 2.2
10. Prochloraz-35	12.1 ± 0.1	4	4	34.4 ± 1.0	82.6 ± 2.0
11. Tebuconazol-12.5	12.5 ± 0.2	8	7	35.9 ± 0.6	90.0 ± 4.2
12. Tebuconazol-50	12.2 ± 0.1	6	6	35.3 ± 0.6	90.7 ± 3.4
13. Procymidon-12.5	12.2 ± 0.1	7	7(#2/2)	34.0 ± 0.7	86.5 ± 3.4
14. Procymidon-50	12.4 ± 0.2	4	4	34.5 ± 0.5	87.1 ± 4.5
Contamed 1					
1.Control	12.4 ± 0.1	14	12(#4/3)	32.5 ± 0.8	98.1 ± 3.2
2. Mix-150	12.1 ± 0.1	13	13(#3/3)	31.5 ± 0.7	81.2 ± 2.8
3. Mix-450	12.5 ± 0.1	14	14(#3/3)	31.4 ± 0.8	80.8 ± 3.2
4. Amix-450	12.6 ± 0.1	14	14(#1/1)	31.5 ± 0.4	80.5 ± 2.3
Contamed 2					
1.Control	12.3 ± 0.1	19	19(#1/1)	34.8 ± 0.6	91.1 ± 2.4
2. Mix-100	12.4 ± 0.1	18	17	33.1 ± 0.5	87.6 ± 3.3
3. Mix-200	12.4 ± 0.1	12	13(#1/1)	31.9 ± 0.4	83.2 ± 2.2
4. Mix-450	12.4 ± 0.1	15	15	33.7 ± 0.5	87.9 ± 2.0
5. Amix-200	12.4 ± 0.1	14	13	33.1 ± 0.4	85.6 ± 2.1
6. Amix-450	12.4 ± 0.1	15	15	34.0 ± 0.3	87.3 ± 1.6
7. Emix-200	12.4 ± 0.1	15	16	33.8 ± 0.4	92.1 ± 2.0
8. Emix-450	12.3 ± 0.1	17	17	33.4 ± 0.5	87.5 ± 2.5
9. Paracetamol-360	12.3 ± 0.1	16	15	33.3 ± 0.5	91.8 ± 2.5
EE2					
1. Control	12.3 ± 0.1	8	8	32.3 ± 1.0	79.0 ± 2.9
2. 5 µg/kg bw/day	12.3 ± 0.1	5	5	33.9 ± 0.5	82.8 ± 5.8
3. 15 µg/kg bw/day	12.3 ± 0.1	9	9(#2/1)	32.0 ± 0.9	77.6 ± 3.1
4. 50 µg/kg bw/day	**12.8 ± 0.2***	8	8(#5/3)	31.9 ± 0.4	74.4 ± 6.6

(#/) Number of animals already showing VO on the first day of registration out of number of litters.* $p<0.01$. NA not assessed.

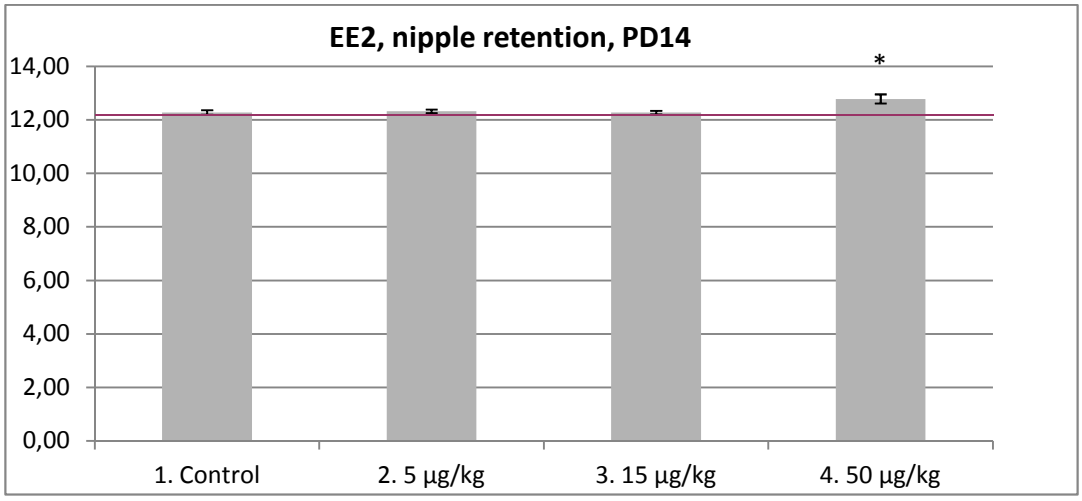


Figure 12. Mean number of nipples ± SEM recorded in female offspring from control group and the groups exposed to EE2. * p<0.01.

4.4 Estrous cycle

Estrous cyclicity was assessed in all studies except for the Pestimix RF study, where the animals were sacrificed before puberty. The results of the estrous cycle measurements performed in the studies are summarized in the tables below.

In the Pestimix DR study no significant effects were detected (Table 9). Most of the observed irregular cycles were longer than normal cycles characterized by an above normal number of days in diestrus. Two animals showed extended estrus (episode of three or four consecutive days of vaginal estrus). There was between 10 and 50% of the animals that apparently had a period of pseudopregnancy. No significant differences were observed between the groups in frequency of pseudo pregnancies.

Table 9. Results of the estrous cycle smear measurements made in 5-6 months old female rats in the Pestimix DR study. The proportion of regular and irregularly cycling animals are shown, and the irregular cycles are further divided into categories according to the type of effect.

Group/study Pestimix	Regular cycling, pct excluding pseudo pregnant rats	Irregularly cycling, pct excluding pseudopregnant rats	Pseudopregnant	Extended estrous	Extended diestrous	Abnormal	Total no. of rats examined	No. of litters	No of litters without pseudo pregnant animals
1. Control	9 (69%)	4 (31%)	5 (28%)	0	1 (25%)	1 (25%)	18	15	12
2. Pestimix-14.58	10 (67%)	5 (33%)	3 (17%)	0	0	3 (60%)	18	17	15
3. Pestimix-29.17	5 (63%)	3 (38%)	2 (20%)	1 (33.3%)	2 (66.6%)	1 (33.3%)	10	8	6
4. Pestimix-43.75	6 (75%)	2 (25%)	4 (33%)	0	1 (50%)	0	12	11	7
5. Epoxyconazol-3.75	5 (63%)	3 (38%)	2 (20%)	0	1 (33.3%)	2 (66.6%)	10	7	5
6. Epoxyconazol-15	3 (60%)	2 (40%)	1 (17%)	0	1 (50%)	0	6	4	4
7. Mancozeb-6.25	6 (100%)	0 (0%)	2 (25%)	0	0	0	8	5	4
8. Mancozeb-25	5 (71%)	2 (29%)	2 (22%)	0	1 (50%)	0	9	7	6
9. Prochloraz-8.75	7 (78%)	2 (22%)	1 (10%)	1 (50%)	0	0	10	9	8
10. Prochloraz-35	3 (75%)	1 (25%)	2 (33%)	0	0	0	6	4	4
11. Tebuconazol-12.5	3 (60%)	2 (40%)	3 (38%)	0	1 (50%)	1 (50%)	8	7	4
12. Tebuconazol-50	2 (50%)	2 (50%)	3 (43%)	0	1 (50%)	1 (50%)	7	7	3
13. Procymidon-12.5	3 (50%)	3 (50%)	2 (25%)	0	1 (33.3%)	1 (33.3%)	8	7	5
14. Procymidon-50	3 (100%)	0 (0%)	3 (50%)	0	0	0	6	4	2

The percentage (pct) of animals in each category is also shown.The percentage of animals shown in the categories extended estrus or diestrus or with abnormal cycle is calculated as percentage out of the number of irregularly cycling animals. Percentage of pseudopregnant animals is calculated out of the total number of rats.

In the Contamed 1 study no significant effects were observed when smears were collected in the 5 or 9-10 months old animals (Table 10). In the five months old animals there were significantly more pseudopregnant animals in the control group compared with Mix-450 and Amix-450 ($p<0.01$ for both groups). No pseudopregnant rats were recorded in the aging rats as this might have been a sign of reproductive senescence rather than pseudopregnancy as both conditions can be characterized by extended periods of diestrus. The use of the VIC device perhaps in concert with taking a swab every day may have caused the large number of pseudopregnancies observed in Pestimix DR and Contamed 1, 5 months old animals. Possible pseudopregnant animals would show up in the table as having abnormal cycles in the older animals (9-10 and 12 months old).

There was a significant effect of exposure in the 12 months old animals on the proportion of animals with an irregular cycle compared to control (Table 10 and Figure 13). Analyzing all the groups the overall p-value was p=0.07, but with a significant trend towards a larger proportion of irregularly cycling animals with increasing dose according to a Cochran-Armitage test (p=0.01). As seen in Table 10 this effect was driven by longer cycles with more than 3 days of diestrus.

Table 10. Results of estrous cycle measurements from Contamed 1 in 5 months, 9-10months and 12 months old rats, respectively. The proportion of regular and irregularly cycling animals are shown, and the irregular cycles are further divided into categories according to the type of effect. A significant trend was observed in the 12 months old animals towards a larger proportion of irregularly cycling females with dosing.

Contamed 1	**Regular**	**Irregular**	**Pseudo pregnant**	**Extended estrous**	**Extended diestrous**	**Abnormal**	**Total no. of rats examined**	**No of litters**
5 months								
1.Control	6 (86%)	1 (14%)	7 (50%)	0	0	1 (100%)	14	12
2. Mix-150	9 (75%)	3 (25%)	2 (14%)	1/3 (33%)	0	1 (33%)	14	13
3. Mix-450	12 (86%)	2 (14%)	0	0	0	0	14	14
4. Amix-450	13 (82%)	1 (18%)	0	0	0	0	14	14
9-10 months								
1.Control	8 (57 %)	6 (43%)	-	-	2 (33%)	1 (17%)	14	12
2. Mix-150	8 (57%)	6 (43%)	-	-	0	4 (67%)	14	13
3. Mix-450	7 (50%)	7 (50%)	-	-	1 (14%)	5/7 (71%)	14	14
4. Amix-450	10 (71%)	4 (29%)	-	-	1 (25%)	1 (25%)	14	14
12 months								
1.Control	9 (69%)	4 (31%)	-	0	2 (50%)	2 (50%)	13	12
2. Mix-150	10 (71%)	4 (29%)	-	0	1 (25%)	2 (50%)	14	13
3. Mix-450	5 (36%)	9 (64%)	-	1 (11%)	4 (44%)	5 (56%)	14	14
4. Amix-450	4 (29%)	10 (71%)	-	0	4 (40%)	5(50%)	14	14

% regularly and irregularly cycling is calculated without the pseudopregnant animals.
Animals are categorized as regularly cycling (4- or 5-day cycles) or irregular ordered in extended (3–4 consecutive days of estrus, or 4–5 days of diestrus), abnormal (>4 days of estrus or >5 days of diestrus). The percentage of animals shown in the categories extended estrus or diestrus or with abnormal cycle is calculated as percentage out of the number of irregularly cycling animals. Percentage of pseudopregnant animals is calculated out of the total number of rats.

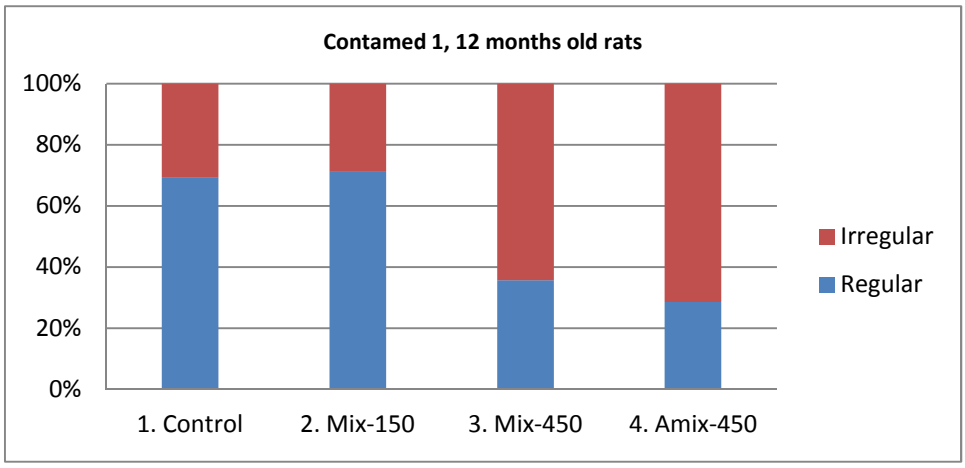


Figure 13. The percent of regular and irregular cycling 12 months old animals in the four groups in Contamed 1. Overall p-value was 0.07 and there was a significant trend (p=0.01).

As can be seen in Table 11 no significant effects were observed in the three months old animals in the Contamed 2 study. In the 12 months old animals there seemed to be an effect in the groups receiving the combined mixtures but this was not significant (p=0.12, when analyzing control , Mix-100, Mix-200 and Mix-450) (Table 11 and Figure 14), however, in the group treated with Mix-200 there was a borderline effect compared with control group (p=0.053). No significant effect was observed when all the groups were included in the analysis and the Cochran-Armitage trend test was not significant (p= 0.07) in the combined mix groups and control only (control, Mix-100, Mix-200 and Mix-450). The irregularly cycling animals generally had cycles characterized by extended periods of diestrus or abnormal cycles, whereas very few showed extended estrus. No animals were observed to be pseudopregnant in Contamed 2.

As the animals in Mix-450 from Contamed 1 and 2 were treated with identical doses, the data for the 12 months old animals were also analyzed together. This lead to a statistically significant difference between control and Mix-450 (p-value was 0.02) (Figure 15).

Table 11. Data from estrous cycle measurements from Contamed 2 at 3 and 12 months of age. No statistically significant effects were observed.

Contamed 2	**Regular**	**Irregular**	**Pseudo pregnant**	**Extended estrous**	**Extended diestrous**	**abnormal**	**Total no of rats examined**	**No of litters**
3 months								
1. Control	15 (83%)	3 (17%)	-	0	3 (100%)	0	18	18
2. Mix-100	17 (85%)	3 (15%)	-	0	1 (33%)	1 (33%)	20	17
3. Mix-200	11 (79%)	3 (21%)	-	0	1 (33%)	0	14	12
4. Mix-450	10 (62.5%)	6 (37.5%)	-	0	3(50%)	2 (33%)	16	14
5. Amix-200	12 (75%)	4 (25%)	-	0	1 (25%)	0	16	13
6. Amix-450	12 (75%)	4 (25%)	-	0	3 (75%)	1 (25%)	16	15
7. Emix-200	15 (100%)	0	1 (6.7%)	0	0	0	16	16
8. Emix-450	12 (75%)	4 (25%)	-	1 (25%)	3 (75%)	0	16	16
9. PM-350	12 (86%)	2 (14%)	-	0	1(50%)	1 (50%)	14	13
12 months								
1. Control	11 (61%)	7 (39%)	-	0	0	6 (86%)	18	18
2. Mix-100	5 (25%)	15 (75%)	-	0	2 (13%)	13 (87%)	19	16
3. Mix-200	**2 (18%)**	**9 (82%)**	-	1 (11%)	1 (11%)	7 (78%)	11	10
4. Mix-450	5 (31%)	11 (69%)	-	0	2 (18%)	9 (82%)	16	14
5. Amix-200	8 (53%)	7 (47%)	-	1 (14%)	1 (14%)	5 (71%)	15	13
6. Amix-450	7 (44%)	9 (56%)	-	0	0	8 (89%)	16	15
7. Emix-200	5 (31%)	11 (69%)	-	3(27%)	3 (27%)	4 (36%)	16	16
8. Emix-450	7 (44%)	9 (56%)	-	0	1(11%)	7 (78%)	16	16
9. PM-350	5 (36%)	9 (64%)		0	1(11%)	6 (67%)	14	13

% regularly and irregularly cycling is calculated without the pseudopregnant animals. Animals are categorized as regularly cycling (4- or 5-day cycles) or irregular ordered in extended (3–4 consecutive days of estrus, or 4–5 days of diestrus), abnormal (>4 days of estrus or >5 days of diestrus). **Bold (p=0.053).** The percentage of animals shown in the categories extended estrus or diestrus or with abnormal cycle is calculated as percentage out of the number of irregularly cycling animals. Percentage of pseudopregnant animals is calculated out of the total number of rats..

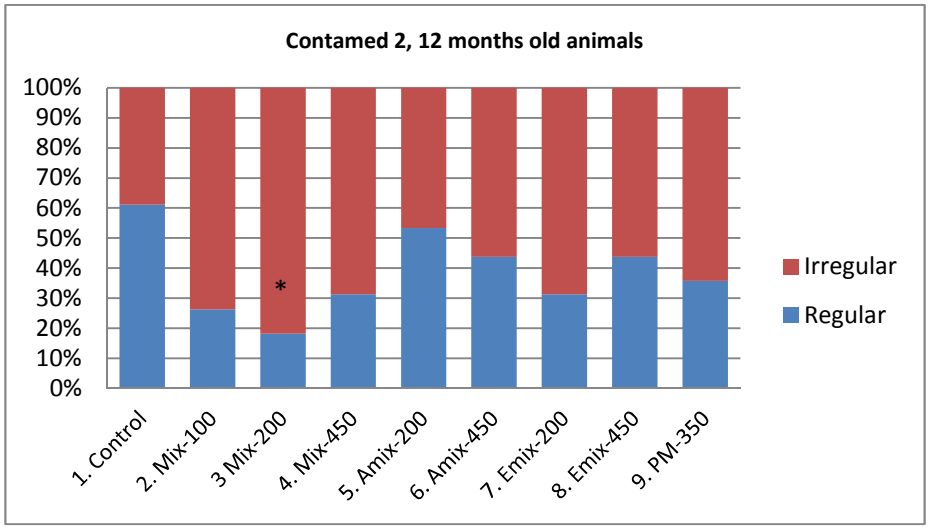


Figure 14. The percentage of regular og irregularly cycling 12 months old animals in the Contamed 2 study. Overall, no significant effects were observed, but comparing only control to Mix-200 the p-value was p=0.053.* p=0.053.

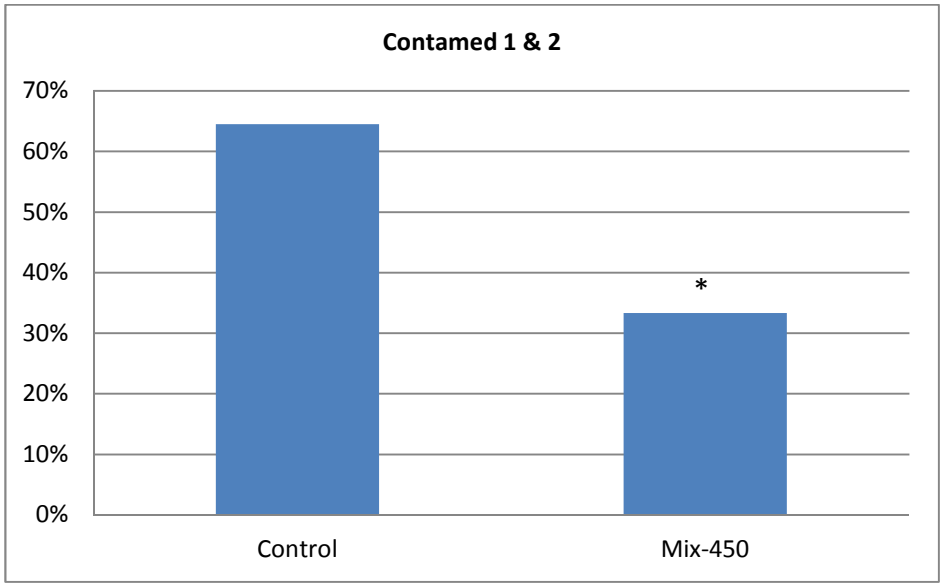


Figure 15. The proportion of regular cycling 12 months old animals. Data is pooled from Contamed 1 and 2.* means p<0.05.

In the EE2 study, as can be seen in Table 12, irregularly cycling females were seen only in the exposed groups; however, there were no statistically significant effects of exposure compared with control group. None of the irregularly cycling animals showed extended estrous (three or more consecutive days of vaginal estrus) and all the long cycles were characterized by an above normal number of days in diestrus. No animals were observed to be pseudopregnant in the period where smears were recorded.

Table 12. Showing the results of estrous cycle measurements in 2.5 months old rats exposed to ethinyl estradiol perinatally. The proportion of regular and irregularly cycling animals are shown. No significant effects were observed.

Study EE2	**Regular cycling**	**Irregular cycling**	**Total no of rats examined**	**No of litters**
1. Control	7 (100%)	0 (0%)	7	7
2. EE2- 5	6 (75%)	2 (25%)	8	5
3. EE2- 15	7 (70%)	3 (30%)	10	9
4. EE2- 50	6 (75%)	2 (25%)	8	7

4.5 Results comparing VIC to reading smear

The use of the Vaginal Impedance Checker (VIC) was assessed as an alternative method to the labor demanding process of collecting, fixating, staining and scoring smears to evaluate estrous cycle status in female rats. True positives were defined as the number of times the VIC correctly identified an irregularly cycling animal as observed in the smear. False positives were the number of times the VIC incorrectly identified an animal as being irregularly cycling. True negatives were the number of times an animal correctly was identified as cycling regularly by the VIC. False negatives were the number of times an animal incorrectly was identified as cycling regularly by the VIC. The data used were from Contamed 1, 5 months old animals including the pseudopregnant, and the results were used to calculate the sensitivity and specificity of the technique as shown in Table 13.

Table 13. The sensitivity and specificity of the Vaginal Impedance Checker

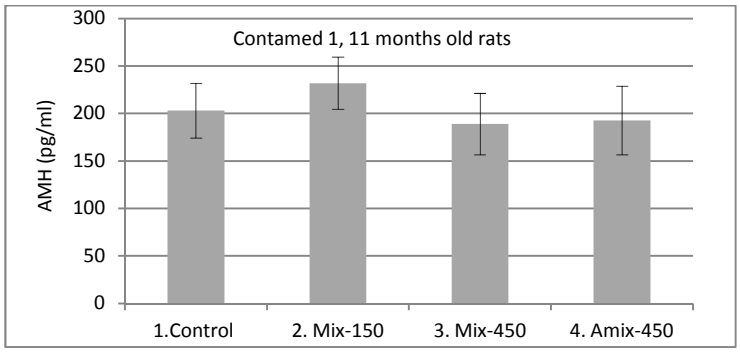
		Gold standard. Smear	
		Condition positive. irregular	Condition negative. regular
VIC	Test outcome pos.	True positive (17)	False positive (24)
	Test outcome neg.	False negative (0)	True negative (15)
		Sensitivity = 1	**Specificity = 0.38**

The sensitivity was calculated to 1 meaning that the VIC identifies all the animals with irregular cycles. A specificity of 0.38 means that 38% of regularly cycling animals is correctly identified as such by the VIC. Thus, the VIC measures too many false positives and no false negatives.

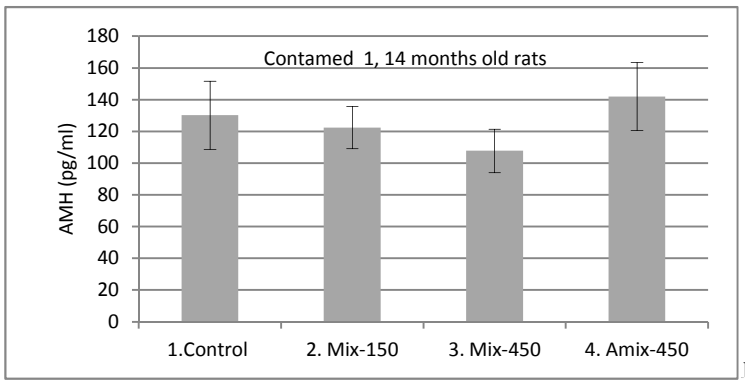
4.6 AMH results

AMH was measured in plasma of female rats at different ages to assess if this endpoint can be of use when investigating effects on the follicle pool of the ovary. The results are shown in the figures 16-19 of this section.

As seen in Figure 16 a and b, no significant effects were observed in the concentrations of AMH in plasma from perinatally exposed females when compared to the plasma levels in the control group in Contamed 1 in 11 months and 14 months old rats.



a)



b)

Figure 16 a) & b). Mean plasma concentration of AMH in 11 months old female rats from Contamed 1± SEM. No statistically significant differences from control were observed when analyzed with or without the outlier in group 4. b) Plasma concentration of AMH in 14 months old female rats from Contamed 1, where data were normalized to an intern standard. No statistically significant differences from control were observed. Data were also analyzed with plate as a random factor.No of litters, n=11-14.

In Contamed 2, 4 months old animals a significant decrease in the level of AMH was observed in the group dosed with paracetamol (group 9) and a decrease was also observed in Mix-450 when all data were analyzed (Figure 17). When only samples measured on the same plate (all the controls, 14/15 in Mix-450 and 11/14 in PM) then the p-value was p=0.01 for both groups compared with control. A somewhat similar picture for Mix-450 was seen in 10 months old rats, however, no statistically significant effect of any of the exposures was found as is shown in Figure 18.

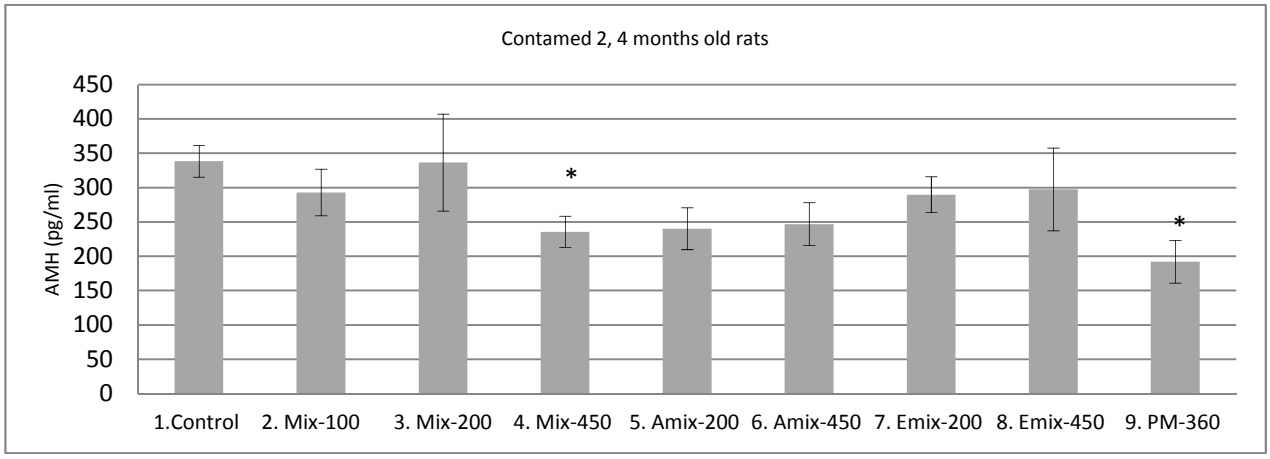


Figure 17. Contamed 2. Mean plasma concentrations of AMH pg/ml ± SEM in four months old female rats. Decreased AMH in group 4 $p<0.05$ and group 9 (PM), *$p=0.02$. No effect on the other groups. Data were analyzed with plate as a random factor. No of litters, n=12-18

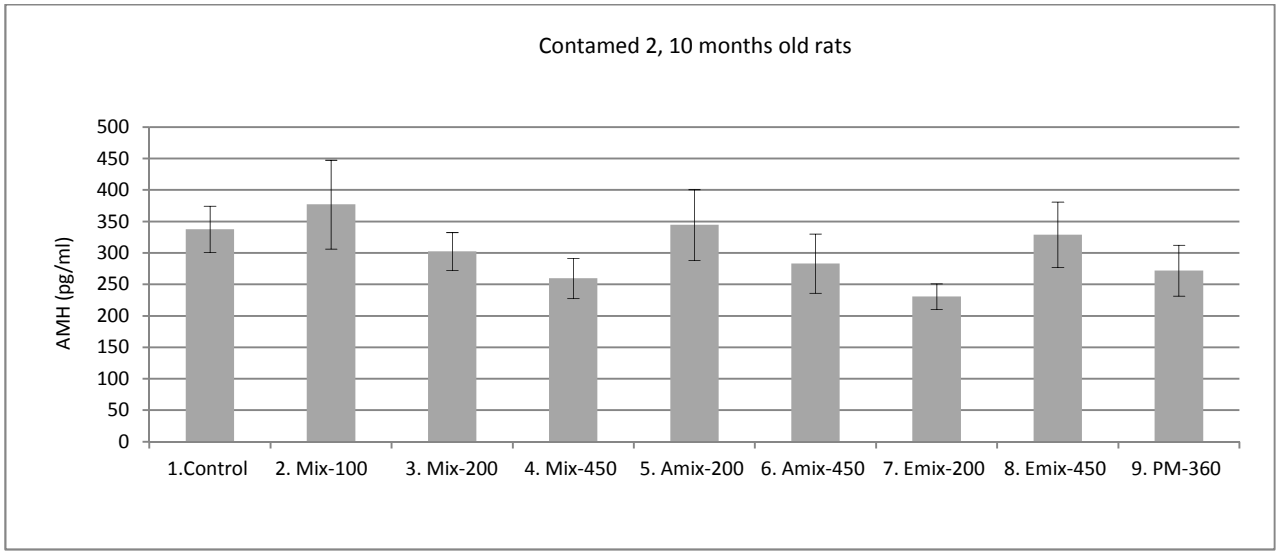


Figure 18. Contamed 2. Mean plasma concentrations of AMH pg/ml ± SEM in ten months old rats. No statistically significant effects on the dosed groups compared to control. No of litters, n=12-17.

There were no statistically significant changes in AMH as a result of exposure to ethinyl estradiol as can be seen in Figure 19.

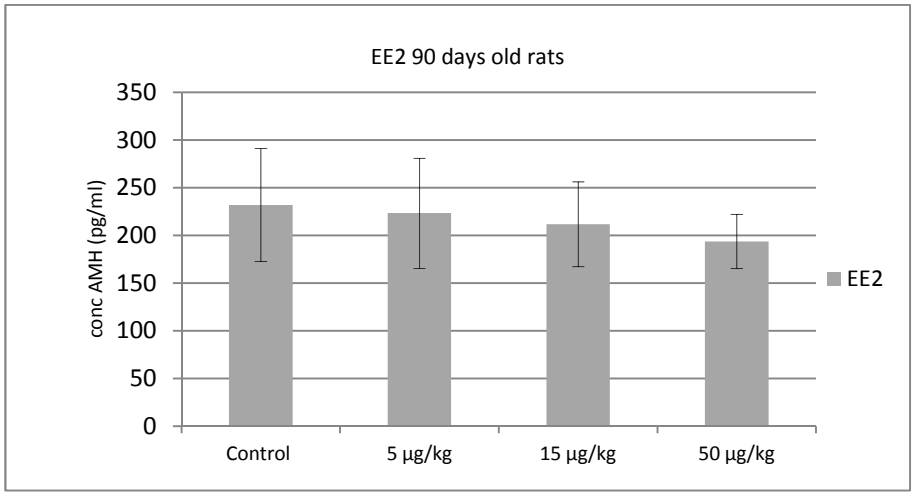


Figure 19. Plasma concentration of AMH (Mean ± SEM) in 90 days old female rats exposed *in utero* to EE2. No of litters, n=5-9. No significant effects were detected.

4.7 Power analysis

Statistical power was calculated on selected endpoints. The power of a test is the probability of not having a false negative outcome.

Estrous cycle data.

The achieved power in these studies varied from 0.26 to 0.75 as can be seen in Table 14. In contamed 2 three months old animals the power is quite low even with 83% regularly cycling animals in the control. The change compared with control, however, in the proportion of regularly cycling animals is also much smaller at three months of age than in for example twelve months old rats in Contamed 2. This affects the power considerably.

Table 14. The table shows the power in the studies using the proportion of regularly cycling animals in the control group and the proportion of regularly cycling animals in the group that differed most from the control.

	study N (total)	**prop regular cycling animals (control group)**	**prop regular cycling animals (dosed group)**	**% change**	**group**	**power in study**
EE2	7-8 (15)	1.00	0.70	30%	15 µg/kg	0.39
Contamed 1(9-10 months)	14 (28)	0.57	0.50	12%	Mix-450	0.57
Contamed 1 (12 months)	13 (27)	0.69	0.29	58%	Amix-450	0.56
Contamed 2 (3 months)	16-18 (34)	0.83	0.63	24%	Mix-450	0.26
Contamed 2 (12 months)	18-16 (34)	0.61	0.18	70%	Mix-200	0.75

N=total number of animals, prop=proportion

As can be seen in Table 15, to detect an effect of 50% depending on the proportion of regular cycling animals in the controls, the total needed group size ranged from around 10 to 50. Again, it seems strange that the data from contamed 2 young animals with a low calculated power gives a lower N than contamed 2 old animals with a higher power. This is because the power in Table 14 is calculated on the basis of the observed proportion of regularly cycling animals in the control group and the group with the highest effect and, as mentioned, there is a much smaller difference between those groups in the young animals (24%) than in the 12 months old (70%). N in Table 15 is calculated with a fixed effect of 20 or 50%, a power of 0.8 and α=0.05. With the larger proportion of regularly cycling animals in the control group of Contamed 2, 3 months old animals, than in the control group of the old animals, it is possible to detect a given effect using fewer animals at 3 months.

Table 15. The table shows the required proportion of regular cyclers necessary in an exposed group, based on the group sizes and control groups in the Contamed studies and the EE2 study, to obtain a power of 0.8, with α=0.05. Furthermore, the table shows the required group sizes were we to detect a 20% and 50% change in the proportion of regularly cycling animals, respectively, with power of 0.8 and α=0.05. N is total sample size.

Study	**total sample size**	**Required prop. Regular cycling in a dosed group**	**N with 20% change (group size)**	**N with 50% change (group size)**
EE2	7-8 (15)	0.37	68 (approximately 30)	21 (approximately 10)
Contamed 1(9-10 months)	14 (28)	0.09	647 (approximately 300)	97 (approximately 50)
Contamed 1 (12 months)	13 (27)	0.09	381 (approximately 150-200)	71 (approximately 35)
Contamed 2 (3 months)	18-16 (34)	0.37	204 (approximately 100)	42 (approximately 20)
Contamed 2 (12 months)	18-16 (34)	0.15	539 (approximately 250)	86 (approximately 40)

AMH data.

G*Power was used to make the calculations to estimate how large group sizes is necessary for the AMH kit to be able to detect an effect of 10, 20 and 50%, respectively. The results are summarized in Table 16. As is apparent from Table 16 the group sizes (number of litters) would range from over 200 pr group with a 10% change to around 15 for a 50% change.

Table 16 The group sizes required to detect a change in AMH levels of 10, 20 and 50%, respectively, with a power of 0.8 and α=0.05. These calculations are based on the normalized data from the 10 months old rats in the Contamed 2 study and the ratio between the numbers of animals there was in the control group and the exposure group in this study as equal group sizes are seldom achieved in these studies.

10% change	N control	343
	N exposed	281
20% change	N control	87
	N exposed	71
50% change	N control	15
	N exposed	13

In Contamed 2, 10 months old animals we would be able to detect an effect around 47% change.

5 Discussion

In the following discussion the endpoints and methods investigated in this thesis will be discussed in more detail. The table below gives an overview of the chemicals tested in the studies included in this thesis and examples of effects that has been reported previously on each endpoint by other investigators. The table is meant to be a starting point for the discussion and give the reader an overview of the effects of the many chemicals. Each endpoint as well as the content of this table will be discussed in the following sections.

Table 17. Features of the chemicals tested in the mixtures or alone in this thesis. The table also shows reported effects from the open literature, on female rodents on each endpoint included in this thesis.

A hyphen (-) denotes cases where values could not be located in the open literature.

Chemical	Use	Mechanisms of action(s)	Gestational length	AGD	Nipples	Estrous	puberty	Estropause
Bisphenol A	Plasticizers	Estrogenic (Salian et al. 2009), *in vitro* AR antagonist (Ermler et al. 2010)	NOAEL > 600 mg/kg bw/day (Tyl et al. 2008)	Longer AGD 2 µg/kg bw/day but not 20 µg/kg bw/day (Honma et al. 2002)	-	Longer cycles at 2 and 20 µg/kg bw/day no NOAEL (Honma et al. 2002)	Early puberty VO, LOAEL: 25 µg/kg bw/day, no NOAEL (Durando 2007), LOAEL: 20 µg/kg bw/day, NOAEL: 2 µg/kg bw/day (Honma et al. 2002).	NOAEL: (50µg/kg bw/day) LOAEL: (50mg/kg bw/day) early irreg. cycles rats (Adewale et al. 2009)
Butyl paraben	Antifungal preservative in cosmetics	Estrogenic (Kang et al. 2002), *in vitro* AR antagonist (Ermler et al. 2011)	NOAEL > 200 mg/kg bw/day (Kang et al. 2002)	NOAEL > 200 mg/kg bw/day (Kang et al. 2002)	-	NOAEL > 1000 mg/kg bw/day (Vo et al. 2010)	100 mg/kg bw/day but not 200 mg/kg bw/day advanced VO (Kang et al. 2002).	-
DBP	Phthalate, used as plasticizers	Inhibitor of testosterone synthesis (Parks et al. 2000, Shultz et al. 2001)	Abortions midterm NOAEL: 250 mg/kg bw/day, LOAEL: 500 mg/kg bw/day (Gray et al. 2006)	NOAEL > 500 mg/kg bw/day (Zhang et al. 2004)	-	NOAEL > 500mg/kg bw/day (Gray et al. 2006)	NOAEL > 1000 mg/kg bw/day (Gray et al. 2006)	-
DEHP	Phthalate, used as plasticizers	Inhibitor of testosterone synthesis (Parks et al. 2000, Wilson et al. 2004)	NOAEL > 405 mg/kg bw/day (Grande et al. 2006, Grande et al. 2007)	NOAEL > 405 mg/kg bw/day (Grande et al. 2006)	NOAEL > 405 mg/kg bw/day (Grande et al. 2006)	Cycle irregular LOAEL: 25 mg/m^3, NOAEL: 5 mg/m^3 (Ma et al. 2006)	LOAEL: 5 mg/m3 (VO advanced) (Ma et al. 2006). VO delayed 2 days. LOAEL 15 mg/kg bw/day. NOAEL: 5 mg/kg bw/day (Grande et al. 2006).	-
Epoxiconazole	Triazole fungicide	*In vitro* multiple mechanisms including anti-estrogenic, AR-antagonist, weak estrogenic activity, inhibit androgen and estrogen synthesis (Kjaerstad et al. 2010)	prolonged gestation NOAEL: 15 and LOAEL: 50 mg/kg bw/day (Taxvig et al. 2007)	Longer AGD. Effect at 15 but not 50 mg/kg bw/day (Taxvig et al. 2007)	NOAEL > 50 mg/kg bw/day (Taxvig et al. 2007)	-	early puberty VO LOAEL: 150 mg/kg bw/day, NOAEL: 100 mg/kg bw/day (de Castro and Maia 2012)	-
Ethinyl estradiol (EE2)	Estrogenic component of contraception pills	Estrogenic (National Toxicology Program 2010)	NOAEL > 50 µg/kg bw/day (Ryan et al. 2010)	Increased AGD, LOAEL: 5 µg/kg bw/day NOAEL: 1 µg/kg bw/day (National Toxicology Program 2010)	-	(cycle irregular) LOAEL: 400 ng/kg bw/day, NOAEL: 4 ng/kg bw/day (Fusani et al. 2007)	Accelerated VO at LOAEL: 5 µg/kg bw/day NOAEL: 0.5 µg/kg bw/day (Ryan et al. 2010)	, NOAEL > 50 ppb (5.8 µg/kg bw/day) (Delclos et al. 2009)

Chemical	Use	Mechanisms of action(s)	Gestational length	AGD	Nipples	Estrous	puberty	Estropause
Linuron	Urea-based herbicide	AR-antagonist (Hotchkiss et al. 2004, McIntyre et al. 2002), inhibitor of fetal testosterone synthesis (Hotchkiss et al. 2004)	NOAEL > 50 mg/kg bw/day/day (McIntyre et al. 2002)	-	-	-	-	-
Mancozeb	Dithiocarbamate fungicide	*In vivo*. affected thyroid function, decreased T_4 levels in pregnant dams, suspected developmental neurotoxicants (Axelstad et al. 2011b, Hurley 1998)	NOAEL > 100 mg/kg bw/day (Axelstad et al. 2011b)	NOAEL > 100 mg/kg bw/day (Axelstad et al. 2011b)	NOAEL 100 > mg/kg bw/day (Axelstad et al. 2011b)	prolonged diestrous, no NOAEL, LOAEL: 500 mg/kg bw/day (Baligar and Kaliwal 2001)	-	-
4-MBC	UV-filter	Estrogenic (Schlumpf et al. 2001), possess *in vitro* AR antagonist potential(Ermler et al. 2011)	no reported effects NOAEL > 100 mg/kg bw/day (Carou et al. 2009)	-	-	NOAEL: 47 mg/kg bw/day (Faass et al. 2009, Schlumpf et al. 2008)	VO advanced LOAEL: 100 mg/kg bw/day (Carou et al. 2009). NOAEL . 47 mg/kg bw/day (Schlumpf et al. 2008)	-
OMC	UV-filter	Estrogenic (Schlumpf et al. 2001), possess *in vitro* AR antagonist potential(Ermler et al. 2011)	NOAEL > 1000 mg/kg bw/day (Axelstad et al. 2011a)	NOAEL > 1000 mg/kg bw/day (Axelstad et al. 2011a)	NOAEL > 1000 mg/kg bw/day (Axelstad et al. 2011a)	NOAEL > 1000 mg/kg bw/day (Schneider et al. 2005)	Delayed puberty NOAEL: 150 & LOAEL: 450 mg/kg bw/day (Schneider et al. 2005)	-
p,p'-DDE	Metabolite of the insecticide DDT	AR-antagonist (Kelce et al. 1995)	NOAEL> 50 mg/kg bw/day (Yamasaki et al. 2009)	NOAEL > 100 mg/kg bw/day (You et al. 1998)	-	NOAEL > 50 mg/kg bw/day (Yamasaki et al. 2009)	Early VO, LOAEL: 50 mg /kg, NOAEL: 15 mg/kg bw/day (Yamasaki et al. 2009)	-
Paracetamol	Analgesic and antipyretic	Inhibitor of prostaglandin synthesis (Kristensen et al. 2011a, Kristensen et al. 2011b)	-	LOAEL: 150 mg/kg bw/day, no NOAEL (Kristensen et al. 2011a)	-	-	-	-
Prochloraz	Imidazole fungicide	AR-antagonist, inhibitor of foetal steroidogenesis (Vinggaard et al. 2006), estrogen receptor antagonist (Laier et al. 2006)	LOAEL: 30 mg/kg bw/day (longer gestation length) and no NOAEL (Vinggaard et al. 2005)	longer AGD in female LOAEL: 50 mg/kg bw/day, no NOAEL (Laier et al. 2006)	-	-	NOAEL > 250 mg/kg bw/day (Noriega et al. 2005)	-
Procymidone	Dicarboximide fungicide	AR-antagonist (Hass et al. 2007, Ostby et al. 1999, Wolf et al. 1999)	NOAEL > 50 mg/kg bw/day (paper II)	NOAEL >50 mg/kg bw/day (paper II)	NOAEL >50 mg/kg bw/day (paper II)	NOAEL >50 mg/kg bw/day Pestimix DR	NOAEL >50 mg/kg bw/day (paper III)	-
Tebuconazole	Triazole fungicide	*In vitro* multiple mechanisms including anti-estrogenic, AR-antagonist, inhibit androgen and estrogen synthesis (Kjaerstad et al. 2010)	prolonged gestation NOAEL: 50 and LOAEL: 100 mg/kg bw/day (Taxvig et al. 2007)	female longer AGD, LOAEL: 100 mg/kg bw/day. NOAEL: 50 mg/kg bw/day (Taxvig et al. 2007)	NOAEL: 100 mg/kg bw/day (Taxvig et al. 2007)	-	NOAEL>60 mg/kg bw/day (Moser et al. 2001)	-

Chemical	Use	Mechanisms of action(s)	Gestational length	AGD	Nipples	Estrous	puberty	Estropause
Vinclozolin	Dicarboximide fungicide	AR-antagonist (Gray et al. 1999, Kelce et al. 1994)	NOAEL > 100 mg/kg bw/day (Schneider et al. 2011)	LOAEL: 100 mg/kg bw/day, decreased AGD PND 14 og 21 (but not AGDI) but there was lower bodyweight. NOAEL: 20 mg/kg bw/day (Schneider et al. 2011)	-	Cycle irregular LOAEL: 100 mg/kg bw/day, NOAEL: 20 mg/kg bw/day, (Schneider et al. 2011)	Advanced, LOAEL: 100 mg/kg bw/day, NOAEL: 20 mg/kg bw/day (Schneider et al. 2011) NOAEL: 56-152 mg/kg bw/day/day (1000ppm) (Matsuura et al. 2005)	-

The table is modified from Christiansen et al 2012 (Christiansen et al. 2012)

5.1 Effects on gestational length

Effects on gestational length are considered an adverse effect in toxicological risk assessment (OECD 2008, U.S. EPA 1996). Gestational length was recorded in all the studies included in this thesis. Compared to vehicle control, gestation length was increased in all of the dosed groups in the Pestimix RF study and in the two highest mixture groups in the Pestimix DR study as well as in the EE2 study in the highest dose group (Table 7).

In the range-finding studies Pestimix RF (Paper I) an effect on gestation length was evident in all exposed groups, including the lowest mixture dose, where each pesticide was present at 25% of the doses that individually previously had not caused effect on gestation length or perinatal pup survival (Table 7). In that study, combined exposure at higher doses induced severe effects manifested as dystochia (impaired parturition) and high perinatal pup mortality (Paper I).

In the Pestimix DR study gestation length was also significantly increased in the two highest mixture groups and in the group exposed to the highest dose of epoxiconazole but not in any of the other single pesticide groups at the doses included in the highest mixture dose. The two mixture doses consisted of each of the pesticides at 17% or 25% of their NOAEL for effect on gestational length based on results of previous studies in our laboratory. These results showed a mixture effect of the pesticides at dose levels where the individual pesticides caused no effects. To my knowledge, this is the first study to describe mixture effects on gestational length.

The mixture effect on the length of gestation is probably due to the presence in the mixture of the three azole fungicides, epoxiconazole, prochloraz and tebuconazole, which have previously been shown to elicit such effects on gestational length (Noriega et al. 2005, Taxvig et al. 2007, Vinggaard et al. 2005, Vinggaard et al. 2006) (Table 17). Prolongation of the gestation period is possibly caused by an increase in progesterone in the dams as suggested for epoxiconazole, prochloraz and tebuconazole (Taxvig et al. 2007, Vinggaard et al. 2005). It has previously been shown that all three azoles are able to increase progesterone levels *in vitro* (Kjaerstad et al. 2010). As a decline in progesterone levels in the dams is a precondition for on-time delivery in rats as mentioned in section 2.2, this may be the cause of the increased gestation length *in vivo*. In agreement with the finding in this study, neither mancozeb nor procymidone have previously been shown to affect gestation length or perinatal survival at the doses studied (Axelstad et al. 2011b, Metzdorff et al. 2007).

In the Contamed 1 and 2 studies no effects were observed on gestational length even though chemicals were included in the mixture which can affect gestational length. The chemicals in the mixtures which has earlier been shown to elicit such effects, but at higher doses, are epoxiconazole and prochloraz (effects observed at

15 mg/kg bw/day and 25 mg/kg bw/day, respectively) (Taxvig et al. 2007, Vinggaard et al. 2005) Taxvig) (Table 17). In comparison the dose of epoxiconazole and prochloraz was 4.5 mg/kg bw/day and 6.3 mg/kg bw/day in the highest mixture, respectively. Paracetamol can possibly affect the process of giving birth as it inhibits prostaglandin synthesis (Christiansen et al. 2012, Kristensen et al. 2011a). It is plausible that the concentration of the chemicals in the mixtures were too low to cause a mixture effect in this study.

In the EE2 study, a prolonged gestational length was observed in the group treated with the highest dose of 50 µg/kg bw/day. No such effect was reported by several other investigators who have dosed rats perinatally with EE2. Ryan et al (2010) dosed Long Evans rats GD7-PND18 by gavage at the doses 0.05, 0.5, 1.5, 5, 15, or 50 µg/kg bw/day and saw no effects on pregnancy length (Ryan et al. 2010). Sawaki and coworkers (2003) dosed Sprague-Dawley rats GD7-PND18 with 0.5, 5, or 50 µgEE2/kg/day and found no effects of exposure on gestation length (Sawaki et al. 2003). In a dietary multi-generation study performed by the National Toxicology Programme (NTP) where Sprague-Dawley rats were exposed to the doses 2, 10 and 50 ppb (roughly corresponding to 0.2, 1 and 5 µg/kg bw/day) no effects on gestation length was seen nor was any effect apparent in their range finding study with doses ranging up to 200 ppb (National Toxicology Program 2010). A possible explanation of the discrepancy between the results reported by these investigators and the finding in the present study may be the differences in the rat strains used as Wistar was used in this study and the strains used in the other mentioned studies was either Sprague Dawley or Long Evans. Since the level of estrogen normally is rising prior to labor onset in rats an estrogenic chemical may not be expected to prolong the gestation period (see section 2.2). The effect observed on gestation length in the group dosed with 50 µg EE2/kg/day in the present study may thus be a chance finding.

5.2 Female AGD

In Pestimix RF a significant effect of exposure to the mixtures compared to vehicle control was evident at the doses where dams gave birth (e.g. Pestimix-43.75 and Pestimix-87.5). This effect was consistent with the results obtained in the Pestimix DR study. In that study a dose-dependent increase in AGDI was seen, in the two highest mixture groups compared with controls (Pestimix 29.17 and Pestimix-43.75) (Table 7). A similar effect was seen in female offspring given the low dose level of epoxiconazole, and both doses of prochloraz and tebuconazole, i.e. at doses equal to those included in the highest mixture dose. No effect was seen in female offspring dosed with a four times higher dose of epoxiconazole and it was hypothesized that the finding at the low dose may be a random finding or alternatively, the lack of effect at the high dose may be due to a limited group size. However, the high doses of both prochloraz and tebuconazole did cause increased female AGDI in the present study. The effect on AGD caused by the mixture was probably due to the three azole fungicides as earlier studies in our laboratory have shown similar effects for epoxiconazole, prochloraz and tebuconazole (Taxvig et al. 2007, Vinggaard et al. 2006)(Table 17). The mechanism

underlying the increased AGDI in the females may be the increased progesterone levels in the dams causing virilization of the female genitals (Willingham et al. 2006).

Exposure to the Contamed mixtures did not induce effects on the AGD of female offspring in the Contamed 1 or 2 study. A number of the chemicals contained in the mixture of the Contamed studies have earlier been shown to alter AGD in females (Table 17). ICR mice exposed subcutaneously with 2 µg BPA/kg GD11-17 but not 20 µgBPA/kg had longer AGD at day of birth (Honma et al. 2002) and others also found an increase in AGD in female Sprague-Dawley rat offspring after continuous dietary exposure to BPA (0.02, 0.3 and 50 but not 5 and 500 mg/kg) (Tyl et al. 2002). Longer AGD in female pups (measured at day of birth) has been reported at 15 but not 50 mg/kg bw/day when the pregnant rat dams were exposed to epoxiconazole from GD7-PND16 (Taxvig et al. 2007). Prochloraz has also caused longer AGD in female offspring of rats exposed during gestation to 50 mg/kg bw/day prochloraz (Laier et al. 2006). In one study vinclozolin has been shown to decrease AGD in female offspring when measured PND14 and 21 at 100 mg/kg bw/day. Anogenital index was not affected, indicating that the AGD decrease was secondary to the lower pup body weight (Schneider et al. 2011). The doses contained in the Contamed studies were lower than the doses of those chemicals that have earlier been shown to affect AGD except for BPA (Table 3 and Table 17). The reason no effects were observed in the Contamed studies with a content of BPA between 0.15 and 0.675 mg/kg bw/day in the mixture, may be the longer, continuous dosing in the rat study or differences in rat strains (Tyl et al. 2002) and in the mouse study differences in sensitivity between Wistar and the mouse model used (Honma et al. 2002). It is also possible that the other chemicals in the mixtures may have interacted with the effects of BPA and thereby reducing it.

In the EE2 study a significant increase of AGD was found in the group treated with 50 µg EE2/kg bw/day. Exposure to estrogenic acting compounds like ethinyl estradiol and genistein has been shown to alter AGD in female rats. However, the data are sometimes puzzling as both longer and shorter AGD and sometimes no effect at all has been reported as outcomes of exposure to these chemicals (Casanova et al. 1999, Delclos et al. 2009, Levy et al. 1995, National Toxicology Program 2010). In a multi-generation study, where female Sprague-Dawley rats were exposed continuously in the diet (including gestation and lactation), a significant increase in AGD in the female offspring of the F2 generation was reported at a dose of 50 ppb (about 5 µg EE2/kg bw/day) but not 2 or 10 ppb (and no effect in F1). In the same study the exposure to 50 ppb EE2 in the diet induced a decrease in AGD in the F3 generation compared with control (National Toxicology Program 2010). Interestingly, the same picture was seen by Delclos et al (2009) in another multi-generation study with dietary exposure on Sprague-Dawley rats (Delclos et al. 2009). In agreement with the findings in the present EE2 study Ryan et al (2010) found an increase in AGD of female Long Evan rat offspring exposed GD7-PND18 to 50 µg/kg bw/day EE2 (Ryan et al. 2010). However, in another study with similar

exposure and dosing period in Sprague-Dawley, no effects of treatment were observed in the offspring at 50 µgEE2/kg bw/day (Sawaki et al. 2003). Thus, the results obtained in this study support an increased AGD after EE2 exposure observed by Ryan et al (2010) but the lack of effects seen in the study performed by Sawaki et al (2003) cannot readily be explained but perhaps there is a difference among strains or laboratory technique.

Generally, in the studies included in this thesis, the AGD analyzed with body weight as a covariate seemed to be consistent with AGDI. An exception was the Pestimix DR study where a significant effect on AGDI was detected in Pestimix-29.17 and the low dose of prochloraz and tebuconazole which were not significant when analyzing AGD with weight as a covariate. It is however, possible that the AGD Index can sometimes overcompensate for the effect of body weight as it assumes the pup to be the form of a cube and an animal with a low body weight can sometimes be thin and not necessarily short and therefore have a longer AGD. AGD did not seem to consistently be the most sensitive endpoints in female offspring in the studies in this thesis as disruption of estrous cycle in the one-year-old rats were observed when AGD was unchanged in the Contamed studies (Table 7).

5.3 Nipples

In the EE2 study an increased number of nipples were seen in the highest dose group (50 µg/kg bw/day) compared with the number of nipples found in control animals. There are reports of effects on the number of nipples in females in the open literature and an effect of androgen exposure during development has in some studies resulted in a lower number of nipples in the female offspring (Hotchkiss et al. 2007, Wolf et al. 2002, Wolf et al. 2004). An increased number of nipples in female rats have, to my knowledge, not been reported earlier in the open literature. Furthermore, no effect on number of nipples in female rat offspring following exposure to doses from 0.05 to 50 µgEE2/kg bw/day dosed perinatally has previously been reported (Ryan et al. 2010). NTP did find hypertrophy of the nipples as an effect of ethinyl estradiol exposure (National Toxicology Program 2010). In males nipple anlagen regression is dependent on the action of androgens in the tissue (Barlow et al. 2004, Hotchkiss et al. 2004, Imperato-McGinley et al. 1986, Kratochwil 1977, Ostby and Gray 2004) and it is possible that excessive presence of estrogen somehow induces a higher number of mammary buds along the mammary streak during mammary development. It may also be speculated that some regression of nipple anlagen happens in females as well as in the males which are then counteracted by EE2. The lack of effects on nipple retention in males treated perinatally with EE2 may argue against that explanation, if it is controlled in the same way in males and females (Ferguson et al. 2011, Howdeshell et al. 2008). No effects were observed on the number of nipples in Pestimix DR and the Contamed 1 and 2 studies.

5.4 Onset of puberty in females

Effects on onset of puberty has as mentioned in section 2.4.1 been observed as an effect of exposure to endocrine disrupters.

No effects were seen in age or weight at onset of puberty on female offspring in either the groups receiving the mixtures or the single chemicals in the mixture studies in this thesis. Others have reported effects on onset of puberty of several of the chemicals in the mixtures of Pestimix DR and Contamed 1 and 2 (Table 17). Epoxiconazole, bisphenol A, p,pDDE, 4-MBC, vinclozolin, butyl paraben and DEHP has been found to advance VO (see Table 17). OMC has been reported to delay VO although according to the author the effect was within historical range and the controls were in the lower range of normal age of VO (Schneider et al. 2005). As it is mentioned in section 2.4, puberty is a complex neuroendocrine process and chemicals with different properties may affect timing of puberty. In the studies in this thesis no effects of exposure to these chemicals on onset of puberty were observed, however, the doses of these chemicals were much smaller in the mixture studies than in the studies where effects were observed (Table 17). The exception is bisphenol A where an advanced VO in female offspring dosed *in utero* through subcutaneous exposure of the pregnant Wistar dams with 25 µg/kg bw/day, has been reported (Durando et al. 2007). Other investigators have observed no effects on onset of puberty when Sprague-Dawley rats were exposed perinatally to bisphenol A in higher doses (3.2, 32 or 320 mg/kg bw/day) GD6-PND20 by gavage (Kwon et al. 2000) or observed a delay in VO also at higher doses (but also in animals with lower body weights) e.g. 500 mg/kg bw/day dietary exposure (Tinwell et al. 2002, Tyl et al. 2002). However that may be, no mixture effects were observed on onset of puberty in these studies and it is difficult to know if an effect of bisphenol A was to be expected in the present study set-up.

In the EE2 study no significant effects were seen on the weight or timing of onset of puberty in female offspring. In female offspring, earlier age of VO as well as VO occurring at a lower body weight when compared to controls has been observed following EE2 exposure at 50 ppb (approximately 5 µgEE2/kg bw) in the F1, F2 and F3 generations in a multi-generation study (National Toxicology Program 2010). Ryan et al 2010 found an accelerated age of VO and at a reduced weight at 5 µgEE2/kg bw (NOAEL 0.5 µg/kg bw/day) in female rat offspring exposed GD7-PND18 (Ryan et al. 2010). Therefore, an effect on timing of VO was expected in this study. However, a problem occurred in measuring VO as some animals in the two highest dose groups had already attained VO on the first day of registration and this may thus result in a weaker statistical result. Another issue is group size, as a group size of around 20 litters is recommended for this endpoint to obtain an acceptable power to detect a 2 days shift (Clark 1999). In comparison, our group size was 5-9 litters, so the lack of statistical significance despite a trend to early puberty in these females is not surprising.

A problem that needs to be considered when assessing these data is the fact that some animals had already attained VO when the registration began (even in the control groups in most studies) making the analysis of the data more difficult. In the PestimixDR study we began registering VO on PD 30. This was too late as some animals already had reached VO at that day. In the following studies VO was registered on 27 (Contamed 1&2 and EE2 studies). This age also turned out to be too late as some animals also had reached sexual maturation at that point also in the control groups. For later studies on puberty onset it is therefore recommend that the recordings of VO begin at weaning (PD22) just to get an idea on how early this strain attains VO. This would be especially important when investigating chemicals suspected to give rise to advanced VO.

5.5 Estrous cycle measurements in adult animals

Evaluating estrous cycle is a very useful indicator of chemical insult to the hypothalamic-pituitary-ovarian axis in females. Estrous cycle was therefore measured in all the studies except for the Pestimix RF, where the animals were sacrificed at an early stage.

Estrous cyclicity was not affected by pesticide exposure in the Pestimix DR study, but we did have a problem with our method, as a large proportion of the animals became pseudopregnant (5/18 animals in the control group alone). The abnormal high number of pseudopregnancies may have been caused by excessive stimulation of the cervix by the use of both a cotton swab to obtain smears and the Vaginal Impedance Checker, as stimulation of the cervix can lead to pseudopregnancy in female rats (Goldman et al. 2007). Moreover, in that study the cotton swab was used before the VIC and for that reason the VIC results were not useful. Because of the large variation in the control group (50 % and 69% regularly cycling when including and excluding pseudopregnant females, respectively), the sensitivity for detecting an increased number of animals with irregular cycles was limited.

Of the tested pesticides in Pestimix DR, only mancozeb has previously been reported in the open literature to have affected estrous cyclicity. Mancozeb induced longer cycles characterized by more days of diestrous in females rats dosed in adulthood (Baligar and Kaliwal 2001), however, much higher doses of mancozeb were used (500 mg/kg bw/day bw. and above) than present in the mixture.

In the Contamed 1 and 2 studies no effects of exposure was observed in the animals either at 3, 5 or 9-10 months of age (Table 10 and Table 11). There were, however, more animals in the control group getting pseudopregnant in the 5 months old animals in Contamed 1. This could be a chance finding or perhaps the animals in the highest mixture group (Mix-450) and AMix-450 are somehow less sensitive to cervical stimulation, as no animals in these groups were pseudopregnant. However, in Pestimix DR the

pseudopregnancies were more evenly distributed among groups indicating no such effect in that study (Table 9 and Table 10).

Chemicals with diverse modes of action have been shown to affect estrous cycle in rodents as mentioned in section 2.5.2. The chemicals contained in the mixture that have been shown to affect estrous cycle of female rodents are mancozeb (LOAEL 500 mg/kg bw/day, (Baligar and Kaliwal 2001), vinclozolin (LOAEL, 100 mg/kg bw/day, (Schneider et al. 2011)), DEHP (LOAEL 25 mg/m3, (Ma et al. 2006)) and BPA (LOAEL, 2 µg/kg bw/day, (Honma et al. 2002)). These chemicals were not present in the mixtures at concentrations which showed a significant effect in any of the exposed groups as compared to controls (Table 3 and Table 17). The exception of this is BPA which in a study has shown effects on estrous cycle in offspring of female ICR/Jcl mice at doses of 2 and 20 µg/kg bw/day (Honma et al. 2002). Four and six months old Sprague-Dawley rats exposed to 1.2 mg/kg BPA bw/day in drinking water GD6-PND22 showed signs of alteration of the reproductive function as measured by the proportion of animals with regular/irregular cycles. The vaginal cytology of some animals revealed intermittent extended periods of diestrus, whereas others exhibited extended periods of proestrus and/or estrus (Rubin et al. 2001). In the Contamed studies effects were not observed at these ages on the estrous cycle with a content of BPA between 0.15 and 0.675 mg/kg bw/day in the mixture and perhaps some of the other chemicals in the mixture somehow counteracted the effect or the Wistar rat strain is not as sensitive as ICR/Jcl mice or Sprague-Dawley to these effects. It is known that the Sprague-Dawley strain starts having irregular estrous cycles at an early age (approximately 6 months) compared with other strains (Eldridge et al. 1999).

In the EE2 study estrous was monitored for 21 days when the female offspring were 2.5 months old. No effects on estrous cyclicity were observed of EE2 exposure compared with control. Others have shown effects of EE2 exposure on the estrous cycle of rodents. In a multi-generational study with continuous dietary exposure in Sprague-Dawley rats, in generation F1 and F2 an increased length of cycles and an increase in number of abnormal cycles were observed due to increased duration of estrus in the groups receiving 50 ppb (about 5 µg/kg bw/day). Smears were taken starting 3 days after VO and for 14 consecutive days. Furthermore, in the F1 generation the group exposed to 2 (0.2 µg/kg bw/day) or 10 ppb (1 µg/kg bw/day) had increased cycle length and animals dosed with 10 ppb had decreased time spent in estrus. The effect on estrous cycle was absent in F3 where exposure was attenuated PND21 (National Toxicology Program 2010). In another study where Sprague-Dawley rat dams were exposed orally by pipette GD5 to PND21and the offspring from weaning to PND32, a larger proportion of irregular animals characterized by persistent estrous were seen at 4 ng/kg bw/day when the animals were around 5.5 months old (Fusani et al. 2007). In a study where Sprague-Dawley rats were exposed to EE2 in utero and postnatally continuously through dietary exposure, effects on estrous cycle (examined for 14 consecutive days starting 3 days after vaginal opening)

by means of increased cycle length and prolonged estrous was seen at 50 ppb (corresponding to 5.8 µg/kg bw/day) (Delclos et al. 2009).

In the EE2 study no effects on estrous cycle were observed but we did have a rather small number of animals which meant that we were not able to detect an effect of less than 50% change in animals with irregular cycles compared with the control group (Table 15). On the other hand the control group had an unusually low number of irregularly cycling animals for our laboratory. Furthermore, our animals were only dosed until weaning PD22 and in most of the above mentioned studies where effects on estrous cycle has been observed the animals have been exposed continuously. Also, again, there may be a difference in sensitivity between the rat strains used.

5.6 Estrous cycle measurements in the one-year-old animals

The processes of reproductive aging involve the hypothalamic-pituitary-ovarian axis. These processes can be disrupted by EDCs and there is evidence of mechanisms involving both effects in the ovary and reprogramming of the hypothalamus as an effect of EDC exposure (Adewale et al. 2009, Armenti et al. 2008, Gore et al. 2011). One of the signs of the progression of reproductive aging are getting irregular cycles and eventually stop cycling altogether (acyclic). Estrous cycles can be measured in rats and used as a sign of possible premature reproductive senescence.

In the 12 months old animals in the Contamed 1 study, there was an effect (although not statistically sigificant) on the number of animals showing irregular cycles with more animals being irregular in the exposed groups dosed with Mix-450 and even more pronounced in AMix-450. Moreover, in Contamed 1 there was a significant trend according to a Cochran-Armitage test. In Contamed 2 there seemed to be an effect on the groups treated with Mix-100, Mix-200 and Mix-450 but the effect was not statistically significant when data from control and the mixture groups Mix-100, Mix-200 and Mix-450 were analyzed together. When comparing the control group with the group treated with Mix-200 a borderline effect was observed ($p=0.053$). However, a significant effect was observed when data from control groups and Mix-450 from both the Contamed 1 and 2 studies were pooled and analyzed together supporting an effect of exposure to the mixture and perhaps reflecting a low power in the studies when analyzed separately (Table 14). The larger proportion of irregularly cycling animals in the exposed groups may be indicative of the animals going into an earlier estropause as an effect of exposure to the mixture. It did not seem as the mixtures only consisting of the antiandrogens, the estrogens or paracetamol, respectively, consistently affected the proportion of irregularly cycling animals, suggesting a mixture effect of both the estrogens and the antiandrogens together. Only BPA was found to have been investigated earlier in regard to early onset of irregular cycles (Table 17). In this study Long Evan rat pups were dosed subcutaneously with either 50 µg

BPA/kg or 50 mg/kg bw/day neonatally PND0-PND3. They found animals exposed to 50 mg/kg bw/day stopped cycling regularly at an earlier age than animals in vehicle control (Adewale et al. 2009).

5.6.1 Early exposure and late effects

As is outlined in section 2.1, the process of follicle assembly and the recruitment of primordial follicles in the ovary to enter into growth are thought to be essential for duration of reproductive lifespan (Abbott et al. 2006, Crain et al. 2008, Diamanti-Kandarakis et al. 2009, Uzumcu and Zachow 2007). Any chemical interfering with these processes may advance timing of menopause/estropause. The levels of androgens and estrogens during development are important as the development of follicles in the ovary is sensitive to increased levels of androgen and estrogens during certain developmental stages and an effect of the estrogens alone may therefore be expected (Abbott et al. 2006, Uzumcu and Zachow 2007). Other mechanisms may be involved as for example reprogramming of the HPO axis as shown in rats exposed perinatally to methoxychlor (Gore et al. 2011). Of the chemicals tested in the Contamed 1 and 2 studies, most of them have been shown to (at least *in vitro*) affect either estrogen or androgen actions Table 17 or has been shown to be able to interfere with folliculogenesis or follicle health in the ovary. Chemicals in the Contamed 1 and 2 studies that have been shown to interfere with folliculogenesis or follicle health in the ovary includes DEHP (Wang et al. 2012), mancozeb (Baligar and Kaliwal 2001), vinclozolin (Matsuura et al. 2005), DDE (Chedrese and Feyles 2001, Crellin et al. 2001) and BPA (Adewale et al. 2009, Suzuki et al. 2002). Thus, a mixture effect on the ovaries resulting in early reproductive aging of the chemicals tested in Contamed 1 and 2 may be plausible but this is of course speculative and need more investigation.

Menopause is a stage associated with considerable health drawbacks and taken together with the fact that women are getting children at a late age the effect observed in rats should be considered a severe effect (see section 2.6.1). The effect on estrous cycle was only evident in the 12 months old rats, and normally the animals are sacrificed long before the age of 12 months in reproductive toxicity studies. Effects late in life are very rarely studied in experimental animals and never after exposure during development in the regulatory test guidelines used for risk assessment. None of the reproductive toxicity studies in the OECD test guideline program (Two-Generation Reproduction Toxicity Study (OECD TG 416) or the extended one-generation reproductive toxicity study (OECD TG443)) are able to address effects in old animals and therefore this type of effects may be overlooked (OECD 2001, OECD 2011). In the Contamed 1 study the exposure caused early endocrine disrupting effects such as increased nipple retention and reduced prostate weights in male offspring at the same dose levels and lower (Paper IV). The effects found in the senescent animals are therefore not necessarily expected to lead to a lower NOAEL, but certainly underscore that severe adverse effects of exposure in early development can be observed late in life.

5.7 AMH

Since the serum levels of AMH has been shown to correlate with follicle pool in rats (Yeh et al. 2007) it could have the potential to be used as an endpoint in toxicological tests when assessing exhaustion of follicle reserve due to chemical insult. Indeed, it has been used as a biomarker to assess the degree of ovarian damage caused by exposure to the chemotherapeutic agent cisplatin (Yeh et al. 2006). Cisplatin is a very potent chemical and it must be assumed that most endocrine disrupting chemicals exerts much more subtle effects on the follicle pool. If the AMH-measurement is to be a useful biomarker of effects on ovarian follicle pool of endocrine disrupters, it could be argued that it must be able to detect the effects caused by less potent chemicals than Cisplatin.

In Contamed 2 a decreased AMH level compared with control was observed in animals exposed to PM and Mix-450 in 4 months old animals (Figure 17). It is striking that there was an effect in group Mix-450 but not in the group exposed to only the highest dose of antiandrogens (Amix-450, group 6) not including paracetamol suggesting paracetamol may be the cause of the decrease in AMH.

No effects were seen in Contamed 1 or 2 in the one-year-old animals, but we did not measure AMH in four months old animals in Contamed 1, so maybe the effect is more pronounced at an earlier age. On the other hand this could be a chance finding as no intern standard was included in the analysis of AMH in the four months old animals in Contamed 2. Notwithstanding this, analyzing data from only the plate where most of the data had been measured did support a significant effect in the groups dosed with Mix-450 and Paracetamol.

In the EE2 study no significant effects of treatment were observed on the levels of AMH. Effects on the ovaries including degeneration of antral follicles (200 ppb, dietary exposure), a lower number of growing follicles in F4 (50 ppb), but a higher number of antral follicles in F1 (10 ppb) was observed in a multi-generational study (National Toxicology Program 2010). In another study where rats were exposed with EE2 GD7-PND21 by gavage, abnormal estrous cycle in 6/8 animals at 6 months of age was observed in the group dosed with 50 µg/kg bw/day EE2 compared with none in vehicle control. Of the animals with abnormal cycles 4 had persistent estrus and these animals also had follicular cysts in the ovary and absence of corpora lutera (Sawaki et al. 2003). According to the authors, these ovarian dysfunctions most likely represent reproductive senescence or a defect in the HPO axis (Sawaki et al. 2003). If there is an effect of EE2 on the follicle pool of the ovary, as these studies may indicate, it may be possible to measure a difference in AMH levels compared with AMH levels in vehicle controls. Looking at the Figure 19 it seems as there could be a trend towards a decrease in the AMH levels with EE2 exposure but this is non-significant. The group sizes in this study only ranged from 5-7 litters and it would have been interesting to investigate if there in fact is an effect of EE2 on AMH levels in a larger study at different ages of the female offspring.

It is known that deficiencies of estrogen and excess of both estrogen and androgens during sensitive windows in development can reduce the number of follicles in the ovary (Abbott et al. 2006). Also, more direct toxic effects causing increased apoptosis in the ovary can affect AMH levels and number of follicles (Yeh et al. 2006) but also chemicals with other modes of actions can disturb the process of folliculogenesis as described in section 2.1 and Figure 2. Thus, theoretically, at least some of the chemicals tested in the studies in this thesis may be able to affect the AMH levels (Table 17).

More work on both the method of measuring AMH and a more focused design needs to be used in order to judge if AMH is a good marker of early reproductive senescence in rodents as it seems to be in humans (section 2.7). These studies were not designed for assessing AMH as a biomarker so the information that comes from them is limited by that. It would in that regard be fruitful to examine both the AMH levels and the follicle counts in the ovaries to verify if any effect on the number of follicles indeed has occurred in groups exposed to an endocrine disrupter known to affect reproductive senescence. If it turns out that an effect decreasing the ovarian follicle pool can be measured at an early age using AMH, this could be a very useful tool perhaps substituting the time consuming smear method which also requires keeping the rats until they are old. More work needs to be done in order to verify the usefulness of this marker.

5.8 Vaginal smear versus Vaginal Impedance checker (VIC)

Vaginal cytology is considered to be the golden standard in staging female estrous cyclicity because the stages can be reliably identified from vaginal smears (Montes and Luque 1988, OECD 2009c). This technique, however, requires technical expertise, are time-consuming and labor-intensive. The rat vaginal impedance checker (VIC), on the other hand, is technically easy to use, less susceptible to subjective interpretation and measurements are very fast to obtain. The theory behind the VIC is that electrical impedance resistance changes during the estrous stages in several species which also includes the rat (Bartos 1977, Ramos et al. 2001, Taradach 1982).

In our study the VIC did not seem to be a good substitute for vaginal cytology when assessing estrous cycle in the rat (Table 13). The VIC was too imprecise even though it had a sensitivity (the proportion of true positives, where positives were defined as the irregular cyclers) of 100%, however, it also had a specificity (proportion of true negatives where negatives were defined as the regular cyclers) of only 38%, resulting in too many animals ended up being categorized as irregular cyclers when compared to visually scoring vaginal smears. Furthermore, there seemed to be a connection between the use of the VIC and animals getting pseudopregnant. Pseudopregnant animals were primarily observed in the Pestimix DR and Contamed 1 where both the VIC and cotton swabs measurements were performed. This could be caused by excessive stimulation of the cervix by the use of both a cotton swab to obtain vaginal smears and the VIC, as stimulation of the cervix can lead to pseudo pregnancy in female rats (Goldman et al. 2007) and has also

been observed by others using impedance measurements (Singletary et al. 2005). In the analysis the assumption was made that the results of reading the vaginal smears were the true answer or the golden standard. It is of course possible that some error has occurred when reading the smears and possibly measuring the hormone levels, which changes during the estrous cycle (see section 2.5.1), would have provided some extra accuracy in determining the cycles of the animals (Butcher et al. 1974). Others have reported peak impedance in either proestrus or estrus or both in the rat (Bartos 1977, Ramos et al. 2001, Singletary et al. 2005) and Singletary et al (2005) suggest the use of vaginal impedance may be limited for chronic estrous cycle monitoring because of the possible risk of inducing pseudo pregnancy (Singletary et al. 2005).

5.9 Power calculations

Power in statistical analysis is the probability of the test will reject the null hypothesis when the null hypothesis is false. The calculated power depends on the sample size used in the studies, the chosen α and the effect (Cohen 1992) which in the case of estrous cycle data is the distribution of regular to irregular cycling animals in the control group compared to the distribution in the group which the control is being compared with. Generally, the power was low when considering the estrous data in the studies included in this thesis. Power ranged between 0.26-0.75 (Table 14) and it is generally considered acceptable that power should be 0.8 or greater (Lipsey and Hurley 2009). When calculating the number of animals necessary to measure an effect on number of regularly cycling animals with a power of 0.8, it is obvious that with the distribution of irregularly cycling animals we have in the control groups in our studies, we would have to have either quite large sample sizes or a large effect in the exposed groups to find an effect. From Table 15 it is seen that it takes sample sizes between 30 and 300 to find an effect of 20% more animals with irregular cycles and 10 to 50 animals per group to detect a change of 50% with the control groups in our studies. In other animal studies performed by other groups the proportion of irregularly cycling animals in the control group has sometimes been lower than in our studies (Faass et al. 2009, Laws et al. 2000b, Matsuura et al. 2005, Schlumpf et al. 2008), however, it is not seldom to have up to 20-30% irregularly cycling animals in the control females (Cooper and Goldman 1999). In our studies the percentage of irregularly cycling rats ranged from 0% in the EE2 study to 43% in the 9-10 months old rats in Contamed 1. Even in 3 months old animals from the Contamed 2 study, where there should not have been any irregular cycles caused by aging, the proportion of irregular cyclers was 17%. Thus, this endpoint is not very sensitive with the strain used in our laboratory and in this type of study design. In studies where estrous cyclicity is measured before and after an exposure, irregular cyclers are often omitted before the exposure to increase the sensitivity of the measure (Cooper and Goldman 1999, Cooper et al. 1996).

In three months old animals in Contamed 2 the power is calculated based on a control group with 83% regularly cycling animals and only a 24% change as the effect, which is a very small effect and not

significant (Table 14). A small effect would normally require a lot of animals to detect compared with a very large effect, hence the low power (0.26). In the 12 months old animals power is calculated based on a control group with only 63% regularly cycling females but a large effect of 70% compared with control group resulting in a fairly high power to detect this specific effect (0.75). Whereas, the power calculations in Table 14 are based on the "real" numbers in both control group and an exposure group obtained in the studies, the calculation of group sizes (Table 15), N, is based on just the control group and the fixed calculated effects of 20% and 50% and a chosen power of 0.8. Because the control group in the 3 months old animals are comprised of 83% regular cyclers compared to only 63% in the 12 months old animals, the calculated required group sizes when measuring an effect of 50 % are (N=around 20) in 3 months old animals (N=around 40) in 12 months old animals, respectively (Table 15).

When measuring the plasma levels of AMH the variability of the measurements were somewhat high and consequently the required sample sizes to obtain a power of 0.8 were also high (between 15 and more than 200, Table 16). With the particular kit used and the group sizes these studies usually have in our laboratory, the effect on AMH would have to be a decrease of about 50% for us to be able to detect it. In another study where AMH has been measured in serum instead of plasma (but also using their own kit), there seems to be a lower variability (Kevenaar et al. 2006). Even though the kit used in this thesis according to protocol should be able to measure AMH in plasma, perhaps using serum and/or trying another kit with hopefully a lower variability could be a fruitful way to further explore this endpoint.

6 Conclusions and perspectives

In toxicological testing the effects of endocrine disrupters have in most cases been more thoroughly investigated in males than females and experimental evidence on the effects on females of combined developmental exposure to environmentally relevant endocrine disrupting chemicals with antiandrogenic or estrogenic modes of action has been missing. Most of the endpoints investigated on female offspring in this thesis were responsive to early exposure to the endocrine disrupters or mixtures of endocrine disrupters tested in the studies.

Interestingly, some pesticides caused prolonged gestational length in dams and in higher doses dystocia when administered as a mixture, even though each pesticide were present at doses below their individual NOAELs for this endpoint. Some of the pesticides and doses of mixture of pesticides in Pestimix RF and DR increased AGD in the female offspring and so did exposure to the highest dose of EE2. For the first time an increase in number of nipples has been reported in female offspring after EE2 exposure. It is not known at this time whether the effect is real or a chance finding. No effects were observed on puberty onset including the study with the positive control EE2, perhaps caused by including too few animals and beginning the registration of VO too late. Future studies on this endpoint should start recording of this endpoint at an earlier age and using a suitable group size. A very interesting finding in the Contamed studies was an effect on the timing of onset of irregular estrous cycles which was evident only in 12 months old animals and not in the younger animals. This effect may be a sign of early reproductive senescence in the animals exposed to the mixture of antiandrogens and estrogens and is an example of early life exposure causing late effects. The effects were probably caused by both the antiandrogens and the estrogens as no consistent effects were observed in the groups treated only with mixtures of chemicals with either modes of action.

Since earlier and more sensitive markers of effects on reproductive senescence of exposure to endocrine disrupters would be valuable AMH levels were investigated. AMH measurement needs further development to judge whether it can be a useful biomarker in future studies. Power calculations suggested estrous cycle as well as AMH measurements are not very sensitive endpoint as only effects of changes around 50% or more could be detected in our rat strain and the AMH-kit used.

To summarize, the following conclusions can be made regarding the hypotheses proposed in the introduction:

a) Yes, the developing females and gestational length in dams were sensitive to exposure to single compounds or mixtures of environmentally relevant compounds.
b) Estrous cycle effects were observed in 12 months old animals but the endpoint was not quite sensitive enough in studies of the sizes included in this thesis.
c) AMH needs more development to assess if it can be used as a marker of reproductive senescence in female rats.
d) The positive control study showed effects on gestational length, AGD and number of nipples but most likely included too few animals to detect effects on estrous cycle, AMH and onset of puberty.

The results obtained in this thesis indicate that exposure to endocrine disrupters may be able to contribute to effects on the reproductive system in girls and women. The fact that mixture effects of exposure during development were seen in the Pestimix studies both on gestational length and AGD below the NOAELs or close to NOAELs indicate a mixture effect of exposure to these pesticides. Effects on gestational length are considered an adverse effect in humans and changes in AGD indicates the normal development of the female reproductive system has been affected. Furthermore, the effects observed on estrous cyclicity in the Contamed studies may be worrying for some highly exposed population groups because only a fraction of the chemicals we are exposed to were present in these mixtures. These studies support the relevance of taking mixture toxicology into account in risk assessment. Moreover, no OECD guideline on reproductive toxicology covers effects observed in aging animals and this is a limitation in existing guideline studies. More work should be focusing on developing sensitive endpoints in model species designed to detect effects on females both early and late in life and also on elucidating the mechanisms of the adverse effects on reproductive development as for example epigenetic programming.

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Appendix I

Paper I. Jacobsen PR, Christiansen S, Boberg J, Nellemann C, Hass U. Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats. Int J Androl. 2010 Apr;33(2):434-42.

Paper II. Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A, Clemmensen LH, Axelstad M.. Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod Toxicol. 2012 Sep;34(2):261-74. Epub 2012 May 29.

Paper III. Jacobsen PR, Axelstad M, Boberg J, Isling LK, Christiansen S, Mandrup KR, Berthelsen LO, Vinggaard AM, Hass U. Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reprod Toxicol. 2012 Sep;34(2):237-50. Epub 2012 Jun 4.

Paper IV. Christiansen S, Kortenkamp A, Axelstad M, Boberg J, Scholze M, Jacobsen PR, Faust M, Lichtensteiger W, Schlumpf M, Burdorf A, Hass U.. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. Int J Androl. 2012 Jun;35(3):303-16. doi: 10.1111/j.1365-2605.2011.01242.x. Epub 2012 Feb 28.

Paper V. Mandrup KR, Jacobsen PR, Isling LK, Axelstad M, Dreisig K, Hadrup N, Vinggaard AM, Hass U, Boberg J. Effects of perinatal ethinyl estradiol exposure in Wistar rats. (submitted to Reproductive Toxicology).

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ORIGINAL ARTICLE

Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats

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Summary

Risk assessment is currently based on the no observed adverse effect levels (NOAELs) for single compounds. Humans are exposed to a mixture of chemicals and recent studies in our laboratory have shown that combined exposure to endocrine disrupters can cause adverse effects on male sexual development, even though the doses of the single compounds are below their individual NOAELs for anti-androgenic effects. Consequently, we have initiated a large project where the purpose is to study mixture effects of endocrine disrupting pesticides at low doses. In the initial range-finding mixture studies, rats were gavaged during gestation and lactation with five doses of a mixture of the fungicides procymidone, mancozeb, epoxyconazole, tebuconazole and prochloraz. The mixture ratio was chosen according to the doses of each individual pesticide that produced no observable effects on pregnancy length and pup survival in our laboratory and the dose levels used ranged from 25 to 100% of this mixture. All dose levels caused increased gestation length and dose levels above 25% caused impaired parturition leading to markedly decreased number of live born offspring and high pup perinatal mortality. The sexual differentiation of the pups was affected at 25% and higher as anogenital distance was affected in both male and female offspring at birth and the male offspring exhibited malformations of the genital tubercle, increased nipple retention, and decreased prostate and epididymis weights at pup day 13. The results show that doses of endocrine disrupting pesticides, which appear to induce no effects on gestation length, parturition and pup mortality when judged on their own, induced marked adverse effects on these endpoints in concert with other pesticides. In addition, the sexual differentiation of the offspring was affected. This as well as the predictability of the combination effects based on dose-additivity modelling will be studied further in a large dose-response study.

Introduction

Animal laboratory experiments have shown that in utero exposure to endocrine disrupting chemicals (EDCs) including some pesticides can cause adverse effects on male reproductive development (Foster, 2006; Gray *et al.*, 2006; Hass *et al.*, 2007; Metzdorff *et al.*, 2007; Christiansen *et al.*, 2008). Individual pesticides alone have so far not been shown to contribute to adverse human effects at relevant exposure levels. However, some studies indicate increased prevalence of cryptorchidism and decreased penile length in sons of women working as gardeners or living on farms where pesticides have been used (Kristensen *et al.*, 1997; Weidner *et al.*, 1998; Carbone *et al.*, 2007; Andersen *et al.*, 2008).

Many EDCs have been found as mixtures in humans (Blount *et al.*, 2000; Swan *et al.*, 2005), including children (Brock *et al.*, 2002; Swan *et al.*, 2005; Main *et al.*, 2006). Damgaard *et al.* (2006) observed an association between congenital cryptorchidism and the levels of certain organochlorine pesticides in mothers' milk (Damgaard *et al.*, 2006). Earlier, Pierik *et al.* (2004) identified paternal exposures to pesticides and smoking as factors associated with these congenital malformations. These initial

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observations in epidemiological studies points in the same direction as laboratory experiments with oestrogenic or anti-androgenic chemicals in which substantial mixture effects occurred even though each individual chemical was present at low, ineffective doses (Rajapakse *et al.*, 2002; Silva *et al.*, 2002; Hass *et al.*, 2007; Metzdorff *et al.*, 2007; Christiansen *et al.*, 2008, 2009).

Some pesticides such as vinclozolin and procymidone antagonize competitively the androgen receptor (AR) binding of androgens and affect mainly the reproductive development in male offspring (Kelce *et al.*, 1997; Ostby *et al.*, 1999). Other pesticides such as mancozeb and propineb act mainly via disruption of the thyroid hormones and are mainly suspected to disrupt brain development (Hurley, 1998; Hass & Axelstad, Personal Communication).

Our detailed research on prochloraz, combined with studies on other azole fungicides such as tebuconazole and epoxyconazole, indicates that these pesticides have the ability to react through several endocrine disrupting mechanisms, and to induce various endocrine disrupting effects (Vinggaard *et al.*, 2005a,b; Taxvig *et al.*, 2007). We have shown that prochloraz induced anti-androgenic effects in rats in vivo in a Hershberger test as well as in a developmental toxicity study (Vinggaard *et al.*, 2002, 2005a). In addition, our studies show that prochloraz increases gestation length and indicate that prochloraz may also affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain (Vinggaard *et al.*, 2002, 2005a). Both tebuconazole and epoxyconazole increase gestation length and pup mortality and furthermore, these pesticides virilise female pups, and affect steroid hormone levels in foetuses and/or dams (Taxvig *et al.*, 2007).

In this article, we present data from two range-finding studies on the effects of a mixture of five endocrine disrupting pesticides. We selected procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb for our experiments. The choice of these pesticides was motivated by their common use as pesticides and their multiple mechanisms. The main aim of our range-finding studies was to assess whether there would be joint effects on pregnancy length and pup survival when every mixture component was present at doses that individually did not, in our earlier studies, produce observable effects on these endpoints. In addition, the aim was to obtain preliminary data on effects on the sexual development of the offspring to plan a large dose-response study.

Materials and methods

Animals and exposure

Two range finding studies, hereafter referred to as study 1 and study 2, were performed 2 months apart. In both studies, time-mated nulliparous, young adult Wistar rats (HanTac : WH, Taconic Europe, Ejby, Denmark) were supplied at gestation day 3 (GD3) of pregnancy.

The animals were housed in pairs until GD18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 × 27 × 43cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 21.00 PM, light intensity 500 lux, temperature 21 ± 2 °C, humidity 50 ± 5%, ventilation 8 air changes per hour). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) were provided ad libitum.

On the day after arrival (GD4), the dams were pseudorandomly distributed into groups of eight animals with similar body weight (bw) distributions. Mixtures were administered by gavage from GD7 to the day before expected birth (GD21) and from pup day (PD) 1 until PD13. However, as most of the exposed dams in study 1 were unable to give birth, only dams from study 2 were dosed from PD1 to PD13.

The substances used were corn oil (vehicle) (Sigma-Aldrich, Brøndby, Denmark), and procymidone, epoxyconazole, tebuconazole, mancozeb and prochloraz. All chemicals were purchased in a technical quality from VWR- Bie & Berntsen (Herlev, Denmark).

The composition of the pesticide mixture (Pmix) was chosen according to the doses of each individual pesticide that caused no major effects on pregnancy length and pup survival in our earlier studies (Vinggaard *et al.*, 2005a; Taxvig *et al.*, 2007). The animals were dosed with vehicle (control) or 25, 50, 75, 100 or 125% of Pmix (see Table 1). The doses used in study 1 were 75, 100 and 125%, whereas the doses studied in study 2 were 25 and 50% of Pmix. However, the dams dosed with 125% of Pmix exhibited signs of acute neurotoxicity after 2 days of dosing. Consequently, the dose was decreased to 100% of Pmix and the dams were included in the 100% group. The dams were inspected twice a day for general toxicity

Table 1 Doses of the five pesticides in mg/kg/day in the Pmix doses

Pesticide	Pmix-25%	Pmix-50%	Pmix-75%	Pmix-100%	Pmix-125%
Epoxiconazole	3.75	7.50	11.25	15.00	18.75
Mancozeb	6.25	12.50	18.75	25.00	31.25
Prochloraz	8.75	17.50	26.25	35.00	43.75
Tebuconazole	12.5	25.00	37.50	50.00	62.50
Procymidone	12.5	25.00	37.50	50.00	62.50
Pestimix, total	43.75	87.5	131.25	175.00	218.75

The mixture ratio was based on doses causing no effects on gestation lengths for the individual pesticides, i.e. Pmix-100%.

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including changes in clinical appearance (e.g. sedation and tremor). Body weights were recorded on GD4 and daily during the dosing period to monitor a decrease or increase in weight gain and the number of pregnant dams is presented in Table 2.

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

Delivery and post-natal development

In study 1, all control animals gave birth as expected on GD22–23, whereas most of the exposed dams exhibited severe problems with parturition. It was therefore decided to end the study on GD25 and perform caesarean section on animals that had not yet given birth. The dams that had already given birth and their pups were sacrificed the same day. The dams were weighed and decapitated after CO_2/O_2 anaesthesia, uteri were taken out, and the number of live or dead fetuses, resorptions and implantations were registered.

The weights of dams and individual pups were recorded after delivery both in the animals in study 1 which were able to give birth and in all the pregnant animals in study 2. The pups were counted, sexed and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups was not related to the time of conception, but was rather similar to post-natal age as the animals gave birth on GD22–24. Body weight of offspring in study 2 was recorded on PD6 and 13.

Anogenital distance and nipple retention

Anogenital distance (AGD) was measured in the offspring at birth (PD1) using a stereomicroscope. On PD13, all male and female pups were examined for the presence of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Female rats normally have 12–13 nipples.

Section PD13, organ weights and assessment of malformations in male external genitalia

The animals were weighed and decapitated after CO_2/O_2 anaesthesia. Testis, epididymis, ventral prostate, seminal vesicles, and liver were excised and weighed from one male per litter. From one female per litter, the uterus and ovary were excised and weighed. The external genitalia of all male offspring were inspected for genital dysgenesis and scored on a scale from 0 to 3, with the observer being blinded with respect to dose group. The scores were:

Score 0 (no effect): Normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact.

Score 1 (mild dysgenesis of the external genitals): A small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening was observed, estimated 0.5–1.4 on an arbitrary scale. The size of the genital tubercle was decreased.

Table 2 Pregnancy and weight data

	Control[a + b]	Pmix-25%[b]	Pmix-50%[b]	Pmix-75%[a]	Pmix-100%[a]
No. pregnant dams	8	4	8	7	14
Maternal bw gain GD7–21 (g)	83.4 ± 3.8	82.6 ± 3.4	74.4 ± 4.7	75.0 ± 10.0	53.7 ± 6.9**
Maternal bw gain GD7–PD1 (g)	9.3 ± 1.8	4.8 ± 3.5	4.9 ± 3.5	–	–
Gestational length (d)	23 ± 0.0	23.5 ± 0.1*	24.1 ± 0.2**	24.8 ± 0.1**	24.6 ± 0.2**
Pup perinatal mortality (%)	7.7 ± 3.6	15.7 ± 12.9	72.8 ± 10.7**	93.9 ± 2.8**	92.8 ± 4.4***
Birth weight, male pups (g)	6.3 ± 0.2	6.2 ± 0.3	6.0 ± 0.1	–	–
Birth weight, female pups (g)	6.1 ± 0.1	6.0 ± 0.2	5.5 ± 0.03**	–	–
Prostate weight (mg)	5.8 ± 0.4	3.6 ± 0.4*	2.1 ± 0.3*	–	–
Left testis weight (mg)	36.9 ± 1.6	33.3 ± 2.5	33.7 ± 1.9	–	–
Right testis weight (mg)	36.0 ± 2.0	33.2 ± 3.0	33.0 ± 1.6	–	–
Epididymis weight (mg)	23.6 ± 0.5	17.4 ± 1.6*	14.9 ± 1.2*	–	–
Uterus weight (mg)	17.1 ± 2.4	14.0 ± 1.3	12.1 ± 2.0	–	–
Liver weight – male pups (g)	0.64 ± 0.03	0.69 ± 0.09	0.85 ± 0.1*	–	–
Liver weight – female pups (g)	0.61 ± 0.02	0.7 ± 0.06	0.72 ± 0.01**	–	–
Liver weight dams (g)	10.4 ± 0.3	12 ± 0.4	11.2 ± 0.2	–	–

Data represent group means, based on litter mean ± SEM.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$.

Birth weight was analysed using the number of offspring as a covariate; organ weights were analysed using body weight as a covariate.

–, no data because of caesarean section in the groups Pmix-75% and Pmix-100%.

[a]Study number 1; [b]Study number 2; [a + b]The control group is representing both study 1 and 2.

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Score 2 (moderate dysgenesis of the external genitals): The preputial cleft was larger, estimated 1.5–2.4 on an arbitrary scale. The urethral opening was situated half-way down towards the base of the genital tubercle (hypospadias).

Score 3 (severe dysgenesis of the external genitals): The preputial cleft was large, estimated 2.5–3.5 on an arbitrary scale. The urethral opening was situated further than half-way down the inferior side of the genital tubercle to the base of the genital tubercle (hypospadias). At the base of the genital tubercle, a groove extending laterally was observed (similar to control females at PD13).

Hormone analysis

Progesterone were analysed in serum from 1–5 male and 1–3 female pups in 4–5 litters pr. dose group at PD13. Testosterone and estradiol were analysed in serum from 1 to 3 male or 1 to 3 female pups in 3–5 litters respectively. Serum from the pups in each litters were pooled by sex. Testosterone, estradiol and progesterone were extracted from the serum as previously described (Vinggaard *et al.*, 2005b) and the hormones were measured by time-resolved fluorescence using commercially available fluoroimmunoassay kits (PerkinElmer Life Sciences, Turku, Finland).

Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Data with normal distribution and homogeneity of variance were analysed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA. Birth weights were analysed using the number of offspring per litter as covariate and organ weights were analysed using body weight as a covariate.

Anogenital distance data were analysed by the calculated AGD-index, namely, AGD divided by the cube root of body weight. The cube root was used because this converts a three-dimensional end point (weight) into a one-dimensional such as the AGD (Gallavan *et al.*, 1999; Gray *et al.*, 1999).

Analysing the level of demasculinization of male pups, the scores were categorized into a binary variable with scores 0 and 1 (no hypospadias) and scores 2 and 3 (mild and severe hypospadias). Statistical analyses of the effects on level of demasculinization were performed using Fisher's exact test.

The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and θ_{max}, with θ_{max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θ_{max} was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method as in Hass *et al.* (2007). All statistical analyses were performed using the SAS procedure PROC GENMOD (SAS Institute Inc, Cary, NC, USA).

Results

Pregnancy data and post-natal survival

There were no significant effects on maternal body weight gain from GD7–21 and GD7–PD1 in dams exposed to Pmix-75% or lower (Table 2). However, maternal body weight gain from GD7–21 in dams exposed to the highest dose of the mixture (Pmix-100%) was significantly decreased (Table 2).

Gestation length was significantly increased in all dosed groups (Table 2) and 5 of 7 dams in Pmix-75% and 9 of 14 dams in Pmix-100% were unable to give birth and had to be sacrificed on GD25 (Fig. 1a).

The number of liveborn pups significantly decreased and the perinatal pup loss was significantly increased at Pmix-50% and higher when compared with controls (Table 2, Fig. 1b). No effects on birth weight were observed in male pups compared with controls, whereas the female pups exposed to mix-50% had a significantly decreased birth weight (Table 2).

No data are shown on birth weight in pups exposed to mix-75% and mix-100%, as there were too few live pups to asses this end point properly.

AGD and NR

It was only possible to record AGD in a few litters in study 1 as most of the dams were unable to give birth (data not shown). In study 2, the mixture produced dose-dependent changes in AGD index with a significant increase seen in females and a decrease in males (Fig. 2a). Nipple retention was significantly and dose-dependently increased in male pups in both groups exposed to the mixture i.e. Pmix-25% and Pmix-50% (Fig. 2b).

Autopsy, organ weight and dysgenesis PD13

No effects were observed on weight of the testes or the uterus in male and female offspring respectively. Weights

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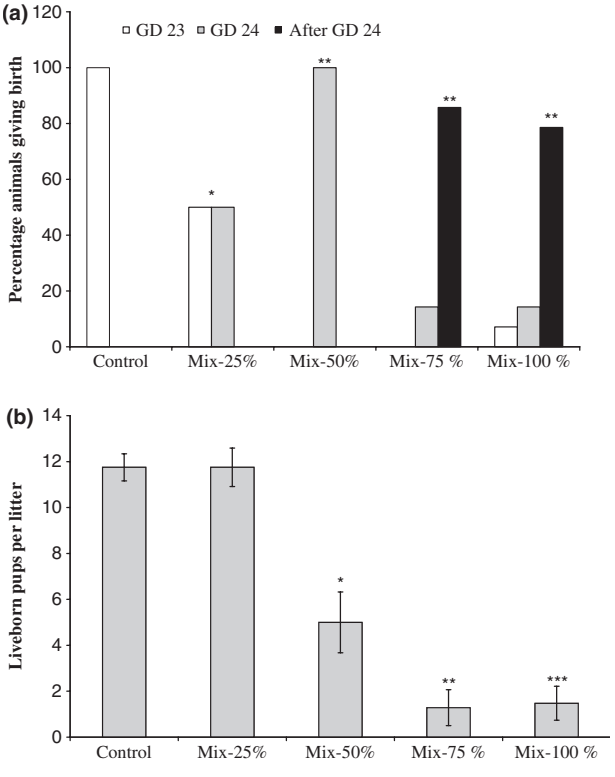

Figure 1 Effects of combined exposure to procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb on parturition (a) and live litter size in rats (b). The parturition results are shown as percentage of animals giving birth on gestation days 23, 24 and after day 24. A few of the animals in the latter group gave birth on gestation day 25, but most of them were unable to give birth. Results shown for number of liveborn pups per litter are group mean ± SD. *p*-values are *<0.05, **<0.01 and ***<0.001. *N* = 8(94), 4(47), 7(40), 7(9) and 14(22) litters (liveborn pups) in Control, Mix-25%, Mix-50%, Mix-75% and Mix-100% respectively.

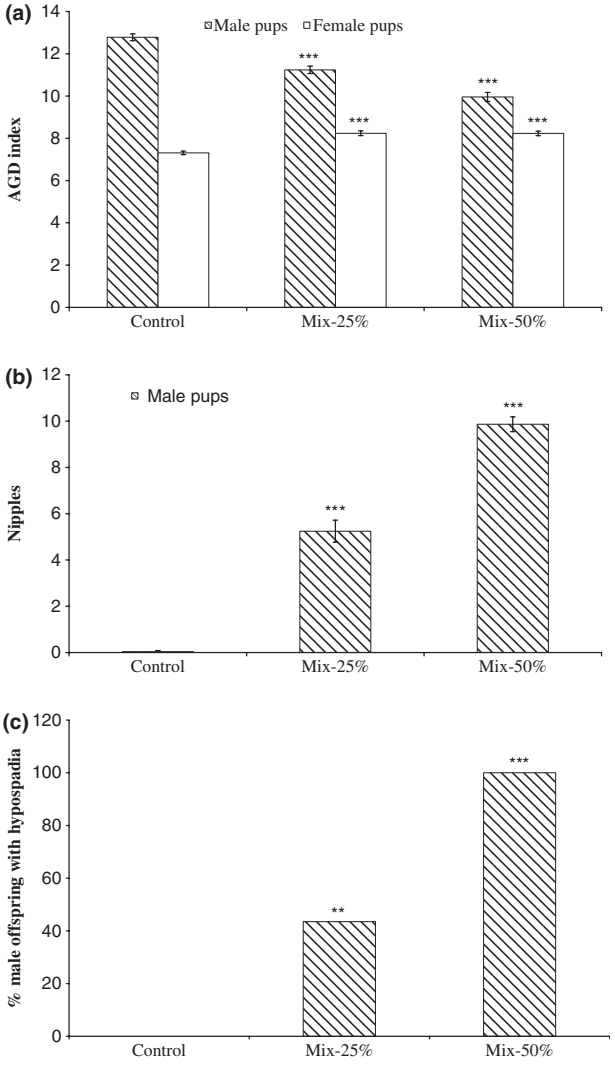

Figure 2 Effects of combined exposure to procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb on anogenital distance (AGD) index at birth (a), nipple retention on pup day 13 (b) and malformation score on pup day 13 (c). Results shown are group mean ± SD. The number of nipples in female controls is generally 12. See text for details on the endpoints. *p*-values are *<0.05, **<0.01 and ***<0.001. *N* = 8(45 : 49), 4(26 : 21), and 7(24 : 15) litters (male : female pups) in Control, Mix-25% and Mix-50% respectively.

of prostate and epididymis in male pups were decreased in Pmix-25% and Pmix-50% exposed animals (Table 2). The liver weights of both male and female pups were elevated in the Pmix-50%-treated animals, but no effects were observed on liver weights of the dams (Table 2).

The incidence of hypospadias was increased with increasing dose (Fig. 2c). In the Pmix-25%, the males had either no, mild or moderate dysgenesis (score 0–2), whereas all of the males in the Pmix-50% group showed severe dysgenesis of the genitalia (score 3). No animals in the control group showed any malformations.

Hormone levels

No statistically significant effects of exposure of Pmix (25, 50%) on progesterone, testosterone or estradiol serum levels were revealed in dams, male or female pups (Table 3). However, large standard deviations and the small number of samples may conceal any real effects.

Discussion

The aim of these range-finding studies was to assess whether there would be joint effects on pregnancy length and pup survival when the five pesticides were present at doses that individually did not produce observable effects on these endpoints in our earlier studies. Moreover, an aim was also to find the dose that should be the highest

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Table 3 Hormone levels in study 2

	Control	Pmix-25%	Pmix-50%
Progesterone levels, males (nM)	0.7 ± 0.4	1.5 ± 0.4	1.3 ± 0.7
Progesterone levels, females (nM)	1.2 ± 0.5	0.9 ± 0.8	0.7 ± 0.2
Progesterone levels, dams (nM)	129.5 ± 44.5	95.0 ± 25.4	82.5 ± 65.6
Testosterone levels, males (nM)	0.3 ± 0.1	0.3 ± 0.2	0.7 ± 0.4
Estradiol levels, females (nM)	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01

Data represent group mean, based on pooled serum ± SD, N = 3–5 litters in each group.

dose in a later large dose response study. Detailed molecular and endocrine analyses were not performed in the current study because of the limited group sizes, but will be targeted in a larger study.

Overall, the findings showed clearly that the combined exposure induced severe effects manifested as dystochia (impaired parturition) and high perinatal pup mortality.

Effect on gestation length was evident in all exposed groups, including the lowest mixture dose, where each pesticide was present at 25% of the doses that individually previously had not caused effect on gestation length. The effect is probably because of the presence of the three azole fungicides in the mixture, which have previously been shown to elicit such effects (Noriega *et al.*, 2005; Vinggaard *et al.*, 2006; Taxvig *et al.*, 2007). The prolonging of the gestation period may possibly be as a result of an increase in progesterone in the dams as suggested for prochloraz, epoxyconazole and tebuconazole (Vinggaard *et al.*, 2005a; Taxvig *et al.*, 2007).

The dystochia and pup mortality seen in the present studies have previously been observed as common effects of several of the azole fungicides (Wolf *et al.*, 1999; Moser *et al.*, 2001; Noriega *et al.*, 2005; Taxvig *et al.*, 2007). Neither mancozeb nor procymidone has earlier been shown to cause effects on pregnancy length or perinatal survival at the doses studied (Metzdorff *et al.*, 2007; Axelstad, Christiansen & Hass, unpublished data from our laboratory). In our large follow-up study, we will aim to avoid dystochia by including the Pmix-25% dose group as the highest exposure group. Another possible way to avoid dystochia without reducing the exposure level would be to stop the dosing of the animals for some days before expected birth. However, as increased pregnancy length is also an important endpoint for endocrine disrupters, we have not chosen this approach. We thereby have also selected the most human relevant dosing period, as pesticides are environmental contaminants that humans can be exposed to throughout pregnancy.

The observed anti-androgenic effects on the sexual differentiation of the male offspring seen as decreased AGD, nipple retention, decreased prostate and epididymis weight and hypospadias are likely because of the combined exposure to the three azole fungicides and procymidone as similar effects to some extent have been seen for the individual pesticides in our earlier studies and by others (Ostby *et al.*, 1999; Wolf *et al.*, 1999; Noriega *et al.*, 2005; Vinggaard *et al.*, 2005a; Laier *et al.*, 2006; Hass *et al.*, 2007; Taxvig *et al.*, 2007; Christiansen *et al.*, 2009).

Prochloraz caused increased AGD at 50 mg/kg and nipple retention at 30mg/kg in male offspring (Vinggaard *et al.*, 2005a; Laier *et al.*, 2006). Genital malformations were observed at 150 mg/kg prochloraz, but no effects on epididymis or prostate were found at the same dose (Laier *et al.*, 2006; Christiansen *et al.*, 2009). Prochloraz is also able to induce increased testicular progesterone concentrations in male rat foetuses (Vinggaard *et al.*, 2005a; Laier *et al.*, 2006; Blystone *et al.*, 2007). Recent studies suggest a previously unidentified role for the progesterone receptor, possibly interacting with the androgen receptor, in disturbed genital tubercle development (Willingham *et al.*, 2006).

Tebuconazole caused nipple retention at 50 and 100 mg/kg/day, whereas epoxyconazole did not induce observable nipple retention at 15 and 50 mg/kg/day (Taxvig *et al.*, 2007). Decreased weight of prostate, but not epididymis, has been observed at 50 mg/kg epoxyconazole, whereas tebuconazole did not affect prostate or epididymis weights up to 100 mg/kg in studies performed in our laboratory (Taxvig *et al.*, 2007).

Procymidone has in our laboratory induced decreased AGD and nipple retention at 25 mg/kg/day, but not at 10 mg/kg/day, while a decreased prostate weight was observed at 10 mg/kg (Hass *et al.*, 2007; Metzdorff *et al.*, 2007). No hypospadias was observed at 25 mg/kg in adult male rats or at 14.1 mg/kg in immature male rats (Metzdorff *et al.*, 2007; Christiansen *et al.*, 2008).

In a similarly designed study in our laboratory, Mancozeb has not shown effects on NR and AGD at doses below 100 mg/kg (Axelstad, Christiansen & Hass, unpublished data from our laboratory).

Thus, the individual doses (Table 1) of each of the three azole fungicides and mancozeb in the pesticide mixture Pmix-25% were clearly lower than those causing no effects on male sexual differentiation, whereas the dose of procymidone was close to this dose level (12.5 mg/kg).

The increased AGD observed in the female offspring is likely to be caused by the combined exposure to the three azole fungicides as our earlier studies have documented similar effects of prochloraz, epoxyconazole and tebuconazole (Laier *et al.*, 2006; Taxvig *et al.*, 2007). This effect may be caused by increased progesterone levels in

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the dams (Willingham *et al.*, 2006). The progesterone as well as testosterone and estradiol levels in the current study were not significantly changed in the dams or in the pups, but this may be as a result of the low number of samples taken in this range finding study with a limited number of litters per group.

Prostate and liver weights were reduced in the present study and it would be relevant to measure changes in gene expression in these organs. In the prostate, androgen-regulated genes such as ornithine decarboxylase and Prostate binding protein subunit C3 (PPB C3) are known to be altered by anti-androgenic compounds (Nellemann *et al.*, 2005). Hepatic expression of growth hormone as well as drug metabolizing enzymes is sexually dimorphic and may be altered by xenobiotics (Waxman & Holloway, 2009). As mentioned above, detailed molecular and endocrine endpoints will be addressed in a later study with more litters per group.

In conclusion, the findings from these range-finding studies showed that combined exposure to the five pesticides induced marked adverse effects on parturition and pup survival at doses where the individual pesticides appear to induce no such effects. The significance of these findings for human risk assessment must be emphasized because they clearly indicate that risk assessment based on single endocrine disrupters alone underestimates the risk for adverse effects when exposure is to several pesticides with common effect outcomes regardless of mechanism.

The sexual differentiation of the offspring was also significantly affected. However, based on a range-finding study with a limited number of litters, only cautious conclusions can be drawn. Consequently, sexual differentiation of the offspring, as well as the predictability of the combination effects based on dose-additivity modelling, is currently studied more thoroughly in a large mixture dose-response study in our laboratory.

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Panel discussion

Ana Soto (Boston, USA)

In your 5 chemical mixture experiment with antiandrogens on fetal rats you observed decreased anogenital distance (AGD) in males and increased AGD in females. What is the mechanism causing this effect in females?

Ulla Hass (Søborg, Denmark)

Not many such studies have been performed in females and the mechanisms are unknown. It is hypothesised that these chemicals in addition to their antiandrogenic effect also have very weak androgenic activity. They bind to the androgenic receptor (AR) but have a very poor stimulatory effect. In males, the binding to AR competes with the much more strongly acting testosterone and therefore the receptor is blocked causing reduced AGD. In the females where there is no testosterone, the weak androgenic activity causes increased AGD. This is one possibility but more research is required and the role of progesterone must also be considered.

Fred vom Saal (Columbia, USA)

Literature from the 1970s described elongated AGD in female rodents developing in utero between two males. The testosterone in the females come from the rodent placenta which, unlike the human placenta, has no aromatase activity and has high levels of C17-20 lyase and 17β hydroxylase: the major sex steroids from the rodent placenta are androstenedione and testosterone, and not progesterone. Pregnant rodent females have serum levels of >1ng/ml testosterone, and 10ng/ml androstenedione. The AGD can be reduced in these masculinised rodent fetuses by antiandrogens such as flutamide or cyproterone acetate. Papers on this finding have been published by Clemens and myself.

Ulla Hass

Thank you for that valuable information which we shall now be assessing.

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Vasantha Padmanabhan (Ann Arbor, USA)

John Marshall has studied the interaction of progesterone and androgen in the human polycystic ovary syndrome. Progesterone is a major negative feedback regulator of LH. High androgen levels such as seen in PCOS can reduce sensitivity to progesterone. A similar scenario may occur in your studies. You are looking at several antiandrogens in unison. Have you plans to assess the response to antiandrogenic with androgenic, or antiandrogenic with oestrogenic EDCs in combination as occurs in real life situations?

Ulla Hass

We are about to test the Contamed mix, which is a mixture of antiandrogen and oestrogen; but not antiandrogen with androgen because I am unaware of environmental pollutants which are androgenic and therefore this might not be of relevance.

Niels E Skakkebæk (Copenhagen, Denmark)

Your hypothesis concerning increased AGD in females is supported by older studies performed in the 1970s using cyproterone acetate (CA) which showed weak virilisation effects on female fetuses when the pregnant dams were exposed to CA.

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Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides

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ABSTRACT

The present study investigated whether a mixture of low doses of five environmentally relevant endocrine disrupting pesticides, epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, would cause adverse developmental toxicity effects in rats. In rat dams, a significant increase in gestation length was seen, while in male offspring increased nipple retention and increased incidence and severity of genital malformations were observed. Severe mixture effects on gestation length, nipple retention and genital malformations were seen at dose levels where the individual pesticides caused no or smaller effects when given alone. Generally, the mixture effect predictions based on dose-additivity were in good agreement with the observed effects. The results indicate that there is a need for modification of risk assessment procedures for pesticides, in order to take account of the mixture effects and cumulative intake, because of the potentially serious impact of mixed exposure on development and reproduction in humans.

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1. Introduction

The high prevalence of disorders related to the endocrine system, *e.g.* hormone-dependent cancers, fertility problems and congenital malformations of reproductive organs, is a point of increasing concern in the western world [1,2]. Many genetic, environmental and lifestyle factors may be involved in these adverse effects, one of them possibly being exposures to endocrine disrupting chemicals (EDCs).

Currently, risk assessment of chemicals including pesticides is based on the no observed adverse effect levels (NOAELs) for effects of single compounds. Based on results from animal studies, exposure to single endocrine disrupting chemicals (EDCs) generally does not cause concern for adverse reproductive effects in humans. Humans are, however, exposed to a mixture of several EDCs [3,4], and during the last decade, scientific and regulatory focus has gradually begun shifting towards examining the effects of mixtures. Since 2005 the European Union member states have for example been obliged to evaluate and if possible refine existing methodologies in order to take combined actions of pesticides into account during risk assessment and especially when establishing maximum residue levels (MRLs) [5].

In studies where experimental animals have been exposed to several endocrine disrupters simultaneously, substantial mixture effects on reproductive development have been seen even though each of the individual EDCs were present at low, ineffective doses [6–8]. In addition, there are indications that cumulative exposure to EDCs including pesticides may play a role in relation to effects on human development, as epidemiological studies have reported increased prevalence of cryptorchidism in sons of women working as gardeners [9] or living on farms where pesticides have been used [10,11]. Furthermore, epidemiological studies have found associations between cryptorchidism and hypospadia and total estrogenic load measured in mother's placenta extracts [12], and association between congenital cryptorchidism and levels of certain organochlorine pesticides in breast milk [13]. Swan et al. [4] found that decreases in anogenital distance among male infants are associated with prenatal phthalate exposure, and Pierik et al. [14]

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identified paternal exposures to pesticides and smoking as factors associated with these congenital malformations. Thus, it is important to bear in mind that exposures to endocrine disrupters may contribute to a combined adverse effect, even though the effects of the single compounds are undetectable [6,8,15]. The findings from animal mixture studies should have major implications for the human risk assessment of EDCs, as they imply that the current use of NOAELs for single chemicals may lead to an underestimation of the potential risk for humans exposed to mixtures of chemicals.

A wide range of pesticides is suspected to act as endocrine disrupters, and there can be many different mechanisms causing the observed effects in animal studies. The five presently investigated pesticides, epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, were selected based on information on pesticide use in Denmark and within the EU, and on their endocrine disrupting activity and effects. Procymidone competitively antagonizes the binding of androgens to the androgen receptor, and thereby mainly affects the reproductive development in male offspring [16,17]. Common features of the azole fungicides epoxiconazole, prochloraz and tebuconazole are that they increase gestational length in dams, virilize female pups and affect steroid hormone levels in fetuses and/or dams [18–20]. Mancozeb acts mainly *via* disruption of the thyroid hormone system and is therefore suspected of disrupting brain development [21,22].

The applied extended developmental toxicity model in rats has previously been employed by our group to characterize the effects of developmental exposure to EDCs on male offspring, and detailed mixture experiments have revealed that statistically significant mixture effects can be observed when anti-androgenic chemicals are combined at levels below their individual NOAELs [6,7]. Before initiating the experimental study presented here, the mixture ratio and dose levels of the individual pesticides were chosen based on earlier obtained dose–response relationships for each of the five pesticides. Hereafter, range-finding studies were performed to find a combined dose that represented the pesticides at doses close to their individual NOAELs, without causing major effects on the pregnant animals or on pup survival. Data from these range-finding studies are presented in Jacobsen et al. [23] and were used to set the dose levels of the mixture and of the individual compounds. Three different doses of the pesticide mixture and two doses of the individual pesticides were investigated in pregnant rat dams, and gestation length as well as anogenital distance, nipple retention and incidence of congenital malformations in the offspring were registered. Based on single chemical dose–response curves obtained in both previous and the present study, the effects of the pesticide mixture were predicted using the mathematical models dose-addition and independent action. Other endpoints sensitive to endocrine disruption, such as reproductive organ weights and histopathology, semen quality and a wide range of neurobehavioral endpoints were also measured in the study, and are presented in Jacobsen et al. [24]. Furthermore, chemical analysis of the pesticides in blood samples from dams and offspring were used for evaluating if mixture exposure and exposure to the single pesticides would lead to comparable blood levels. These results are presented in Herrmann et al. [25].

One goal of mixture toxicology is to estimate the toxicities of untested mixtures of chemicals on basis of information on individual components. For predicting combined effects of the five pesticides, we assumed that each pesticide in the mixture did not exacerbate or diminish the effects of the other pesticides. The choice of an appropriate model for calculation of additivity expectations is essential for assessments of mixture effects, because it is in relation to these additivity expectations that combination effects are judged in terms of synergisms or antagonisms [26]. Mixture effects according to "additivity" assumptions can be calculated by using two alternative concepts, dose addition and independent action. Dose addition looks at mixture effects in terms of a "dilution principle". It assumes that one chemical can be replaced totally or in part by an equal fraction of an equi-effective dose of another, without diminishing the overall combined effect [27]. Dose addition is often used for mixtures composed of chemicals that act through a similar or common mode of action [28–32]. Its application to the present mixture of five pesticides appeared justified because all pesticides are endocrine disruptors and – apart from mancozeb – produce a common effect outcome, *i.e.* anti-androgenic effects in male offspring. However, it can be argued with equal justification that the similarity assumption for dose addition is not applicable to the chosen mixture because the pesticides produce their effects by a diversity of molecular mechanisms. For this reason, we also employed the alternative concept of independent action to estimate additivity expectations. Independent action assumes that the joint effects of a combination of agents can be calculated by adopting the statistical concept of independent events [33]. It is viewed as appropriate for mixtures of chemicals with diverse modes of action [28], however, dose addition and independent action often yield additive mixture effect predictions within the same range.

The overall aim was to explore whether a mixture of environmentally relevant endocrine disrupting pesticides with dissimilar modes of action would cause adverse developmental toxicity effects in rats at dose levels below NOAELs for the individual pesticides. Furthermore, the aim was to investigate whether dose-additivity or independent action predictions of the expected mixture effects resulted in useful estimates compared to the observed mixture effects for relevant endpoints. The mixture and the single pesticides were also investigated using *in vitro* assays, in order to compare these results with those from the rat study, and evaluate the usability of alternative *in vitro* methods for estimating potential mixture effects.

2. Materials and methods

2.1. Chemicals

The five pesticides used were epoxiconazole (CAS no. 106325-08-8, purity 99.0), mancozeb (CAS no. 8018-01-7, purity 76.0), prochloraz (CAS no. 67747-09-5, purity 98.5), tebuconazole (CAS no. 107534-96-3, purity 98.5) and procymidone (CAS no. 32809-16-8, purity 99.5). All chemicals were purchased in a technical quality from VWR-Bie & Berntsen, Herlev, Denmark. Corn oil (Sigma–Aldrich, Brøndby, Denmark) was used as vehicle.

2.2. Animals and exposure

The animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee.

The study included 198 time-mated nulliparous, young adult animals (Han-Tac:WH, Taconic Europe, Ejby, Denmark) distributed to 14 groups of animals. It was performed in 4 blocks with a week between each block and the 14 groups were as equally as possible distributed among the 4 blocks. The animals were housed in pairs until GD (Gestation Day) 18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 cm × 27 cm × 43 cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light–dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature 21 ± 2 °C, humidity 50% ± 5%, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) was provided *ad libitum*. The animals were observed twice daily for signs of toxicity and body weights were recorded daily during the dosing period.

On the day after arrival, at gestation day (GD) 4, the time-mated animals were pseudorandomly distributed into groups with similar body weight (bw) distributions. They were given four days after arrival to adapt to the reversed light-dark cycle before beginning the exposure. Independently of actual day of delivery, the expected day of delivery, GD 23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age. Dams were dosed daily by gavage, from GD 7 to 21 and from the day after birth to pup (PD) 16, and were treated at a constant volume of 2 ml/kg/day, with individual doses based on the body weight of the animal on the day of dosing.

Table 1
Doses of the pesticides administered individually and in mixture to pregnant rat dam from GD (gestation day) 7 to PD (pup day) 16 (in mg/kg bw/day) and number of dosed time-mated rats (dams with viable litters) in each group.

Group	n[a]	Epoxiconazole	Mancozeb	Prochloraz	Tebuconazole	Procymidone	Pesticide mixture
1: Control	22 (15)	–	–	–	–	–	–
2: Pestimix-14.6	22 (17)	1.25	2.08	2.92	4.17	4.17	14.6
3: Pestimix-29.2	22 (9)	2.5	4.17	5.83	8.33	8.33	29.2
4: Pestimix-43.8	22 (14)	3.75	6.25	8.75	12.5	12.5	43.8
5: Epoxi-3.75	12 (8)	3.75	–	–	–	–	
6: Epoxi-15	10 (4)	15	–	–	–	–	
7: Manz-6.25	12 (5)	–	6.25	–	–	–	
8: Manz-25	10 (7)	–	25	–	–	–	
9: Prchl-8.75	12 (8)	–	–	8.75	–	–	
10: Prchl-35	10 (4)	–	–	35	–	–	
11: Tebu-12.5	12 (8)	–	–	–	12.5	–	
12: Tebu-50	10 (6)	–	–	–	50	–	
13: Procy-12.5	12 (7)	–	–	–	–	12.5	
14: Procy-50	10 (4)	–	–	–	–	50	

Epoxi: epoxiconazole; Manz: mancozeb; Prchl: prochloraz; Tebu: tebuconazole; Procy: procymidon.
[a] The large difference between the number of exposed time-mated rats and dams with viable litters was mainly due to unusually low pregnancy rate from the animal breeders and not to high rates of pre- and postnatal mortality in the offspring.

2.3. Mixture composition

To design the mixture of the five pesticides, developmental toxicity data from previous studies with each of the pesticides were used. The mixture ratio for the five pesticides was chosen based on the dose–response relationship and the NOAEL for effects on increased gestation length in dams and perinatal mortality in the offspring in previous studies in our laboratory (Supplemental Material Table X1). Epoxiconazol, prochloraz and tebuconazol cause effects on pregnancy length and perinatal survival. The effects on perinatal mortality at the LOAELs were more marked for epoxiconazol and tebuconazol than for prochloraz. Based on this the doses chosen for epoxiconazol and tebuconazol in the mixture were their NOAELs for these effects, whereas a slightly higher dose of 35 mg/kg/day was chosen for prochloraz. Neither mancozeb nor procymidone caused effects on pregnancy length or perinatal survival at the doses studied. For mancozeb, a dose level of 50 mg/kg/day in the mixture was initially chosen, because our earlier studies have shown that dose levels of 150 mg/kg/day and higher cause marked acute neurotoxicity in pregnant animals [21]. Due to acute neurotoxicity in a range-finding study using non-pregnant animals the mancozeb dose was decreased to 25 mg/kg/day in all of the studies in pregnant animals. The dose level for procymidone was chosen mainly based on the anti-androgenic potency in our earlier studies (Supplemental Material Table X1).

Upon choosing the mixture ratio, two range-finding studies were performed. The first was in non-pregnant animals while the second was in pregnant animals, and they were performed in order to test for toxicity and endocrine disrupting effects of various mixture doses [23]. The highest dose of the mixture, which did not cause maternal toxicity in the range-finding study in pregnant animals, was used as starting point for the present study.

In Table 1 the composition of the pesticide mixture, the doses of the pesticides administered individually and in mixture and the number of litters in each group are shown. Four groups of 22 time-mated rats were given daily oral doses of 0, 14.6, 29.2 or 43.8 mg/kg/day of the mixture of the 5 pesticides, whereas ten groups of 10 or 12 time-mated rats were similarly dosed with two doses of the individual pesticides. The lowest dose of each pesticide was similar to the dose included in the highest mixture dose and the highest dose of the single pesticides was 4 times higher.

2.4. Gestation length, anogenital distance and nipple retention

After delivery, which occurred from GD22 to 24, the weights of dams and individual pups were recorded and the pups were counted, sexed, checked for anomalies and anogenital distance (AGD) was measured using a stereomicroscope. Additionally, anogenital index (AGDI), *i.e.* AGD/cubic root of body weight was calculated for all offspring. Pups found dead were macroscopically investigated for changes when possible. Weight of pups was recorded again on days 6, 13, 22. On PD 13, all male and female offspring were examined for the presence of areolas/nipples (nipple retention, NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring.

2.5. Genital malformations in male offspring

On PD 16 and PD 22, 1–3 male pups per litter were randomly selected for autopsy. On PD 22 the pups were decapitated in CO_2/O_2 anesthesia. Male reproductive organs were examined macroscopically for anomalies and testicular descent and scored for the degree of external genital malformations on a scale from 0 to 3, with the observer being blinded with respect to dose group.

The scores were:

Score 0 (no effect): Normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact.

Score 1 (mild dysgenesis of the external genitals): A small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening was observed, estimated 0.5–1.4 on an arbitrary scale. The size of the genital tubercle was decreased.

Score 2 (moderate dysgenesis of the external genitals): The preputial cleft was larger, estimated 1.5–2.4 on an arbitrary scale. The urethral opening was situated half-way down towards the base of the genital tubercle (hypospadias).

Score 3 (severe dysgenesis of the external genitals): The preputial cleft was large, estimated 2.5–3.5 on an arbitrary scale. The urethral opening was situated further than half-way down the inferior side of the genital tubercle to the base of the genital tubercle (hypospadia). At the base of the genital tubercle a groove extending laterally was observed (similar to control females).

Gubernacular length was determined as a measure of testicular descent, as an increased gubernacular length would indicate cryptorchidism.

All weaned male offspring were scored alive for external genital malformations shortly after sexual maturation (around PD 50) using a similar scoring system, that was modified to reflect the development of the external male sex organs.

At the age of approximately 9 months, the 1–2 males per litter were decapitated in CO_2/O_2 anesthesia, and external genitals were inspected for the following anomalies: descendent testes, alopecia in the perineal area, cleft phallus and hypospadia. Malformations were scored using a system with 0 denoting the normal and 3 the most severe changes. The observer was blinded with respect to dose groups.

2.6. Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means or Dunnett. Birth weights were analyzed using the number of offspring per litter as covariate and AGD was analyzed using body weight as a covariate. Additionally, anogenital-index (AGDI), *i.e.* AGD divided by the cube root of body weight, was analyzed. The cube root was used because this converts a three-dimensional end point (weight) into a one-dimensional such as the AGD [34].

When analyzing the level of dysgenesis or malformations of external male organs the scores were categorized into a binary variable with score 0 (normal) and scores 1, 2 and 3 (mild to severe effect). The results are generally shown both as number of offspring and number of litters affected per group, because malformations are rare events that may only affect very few litters. Statistical analysis based on the litter as unit is therefore rather insensitive and consequently it is current practice also to include offspring data in the toxicological evaluation. The statistical analyses of the malformations were done using Fisher's exact test on litter data.

The number of nipple/areolas was assumed to follow a binomial distribution with a response range between 0 and θ_{max}, with θ_{max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θ_{max} was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipple/areolas and litter were modeled by the Generalized Estimating Equations method as in Hass et al. [6]. The number of nipples in the all control group males was zero and it was therefore necessary to put in 1 nipple in 3 pups from the control group to perform

the statistical model. All statistical analysis was performed using the SAS procedure PROC GENMOD.

Asterisks in tables and figures, indicate a statistically significant difference compared to controls $^{*}p \leq 0.05$; $^{**}p < 0.01$. $^{***}p < 0.001$. All analyses were performed using SAS Enterprise Guide 3.0, SAS Institute Inc, Cary, NC, USA.

2.7. Mixture modeling

The data for the single pesticides comprised the raw data from our previous studies (overview in Supplemental Material Table X1) and the data for both single pesticides and the mixture obtained in the present study.

Endpoints considered relevant for this modeling were those observed to show an effect at mixture dose levels at which the individual pesticides when given alone caused no or significantly smaller effects. These endpoints included gestation length, anogenital distance in female offspring, nipple retention and genital malformations in male offspring. The modeling was, however, not done for the latter endpoint due to lack of sufficient data for the single pesticides.

Data were normalized to the control groups of each year (experiment) so that year did not have to be included in the model as an extra factor. The effect of year was tested and either *t* test or lmein R was used.

The increase in gestation length was normalized to lie between zero and one, where the mean of the control group was the minimum (0) and the mean of the control group plus 2 was the maximum (1).

The endpoint nipple retention (NR) was scaled using the mean of the female controls (approximately 12–12.5). Values larger than this maximum were thresholded to the maximum. After scaling according to the female controls, the controls were not included in the analyses, but they are plotted in the figures as reference points.

The endpoint AGDI in female offspring was transformed for each experiment to lie between zero and one, scaled with the mean of the female controls as the minimum value, and the mean of the male controls as the maximum value.

For NR in males and AGDI in females, the same model and fitting method was used since these endpoints have several observations per litter. First, a binomial logit curve was fitted to the observations for each of the pesticides. Generalized Estimating Equations (GEEs) were used to perform the fit [35–37]. This method is particularly good when accounting for correlations within subject (here litter), but independence across litters, *i.e.* the model is based on pup level but taking the litter effect into account. The variance of the estimates can be incorrect if the correlation structure is not accounted for. In order to keep the number of parameters to a minimum in this model we assumed that the correlation is so called "exchangeable", meaning that it is the same for any pair of litters.

The models were fitted using the function gee [38] in the R package gee from CRAN.

For gestation length, a generalized linear model with a binominal logit (GLM) was fitted to all observations for the five pesticides and the pesticide mixture. As gestation length was measured on dam level, there was only one observation per litter, and therefore a GLM rather than a GEE was used. The glm() function in the statistics program R was used for estimation and prediction.

Either a GEE or GLM was fitted to the same endpoints for both single pesticides and the mixture. Two models for prediction were considered. First, the dose-additivity predictions were estimated. These are defined by the effect dose of the pesticide mixture:

$$ED_{x_{\text{mixture}}} = \left(\frac{p_1}{EDx_1} + \frac{p_2}{EDx_2} + \frac{p_3}{EDx_3} + \frac{p_4}{EDx_4} + \frac{p_5}{EDx_5} \right)^{-1}$$

for the effect doses of the five pesticides in question, and where p_i is the proportion of pesticide i in the mixture. The parametric delta method (closely related to parametric bootstrapping) was performed to estimate the $EDx_{mixture}$ from the fitted models and their variance estimates [39]. The distributions of the parameter estimates were sampled using the estimated variances of the parameters α and β. Random normal samples were used to sample new parameters and thereby obtain predictions using a Monte Carlo sampling strategy assuming a normal distribution of the parameters α and β. A thousand samples were drawn for each parameter, and a dense grid of the doses was used to estimate the effect doses.

Secondly, the independent actions model [33]:

$$E_{x_{mixture}} = 1 - (1 - Ex_1) \times (1 - Ex_2) \times (1 - Ex_3) \times (1 - Ex_4) \times (1 - Ex_5)$$

was likewise estimated using such a Monte Carlo sampling. Here, Ex_i denotes the fractional effect (in %) of the ith pesticide, where we have $x_i = x_{mixture} \cdot p_i$. Note that the independent actions formula is given for the effect whereas the dose-additivity model is given in terms of the effect dose.

The same analyses were also performed with winsorization, as described by Scholze et al. [40]. This means that the fitting was performed iteratively with observations which fall outside 3 times the standard deviation of the mean prediction are adjusted to the value of 3 times the SD and then a new fit is performed *etc.*

2.8. In vitro assays

The pesticide mixture for the *in vitro* studies was designed based on the mixture applied for the *in vivo* studies. The ratio of the 5 pesticides (epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone) was:

	Epoxiconazole	Mancozeb	Prochloraz	Tebuconazole	Procymidone
mg basis	15	25	35	50	50
Molar basis	3	2	6	11	12

2.8.1. Androgen receptor reporter gene assay

The ability of the pesticides to activate the androgen receptor (AR) and to inhibit androgen-induced activation of the AR was tested in an AR reporter gene assay based on AR-transfected Chinese Hamster Ovary (CHO) cells as described by Kjaerstad et al. [41]. The test compounds were tested in triplicates within the range 0.03–30 μM, and luciferase activity was measured as described by Kjaerstad et al. [41].

Cytotoxicity experiments were performed as described by Kjaerstad et al. [41].

2.8.2. T-screen for thyroid receptor activity

The T-screen assay was used for *in vitro* detection of the agonistic and antagonistic properties of the test compounds at the level of the thyroid hormone receptor (TR). The GH3 cells employed in the T-screen assay is a rat pituitary tumor cell line, which growth is dependent on the thyroid hormone (TH) 3,3′,5-triiodothyronine (T3), and which has a high expression of TR. The growth stimulatory effect of T3 is mediated by specific, high-affinity TRs that upon binding of THs bind to thyroid hormone responsive elements (TREs) in the cell nucleus ultimately leading to gene expression and subsequent cell proliferation [42]. The rat pituitary cell line GH3 were obtained from American type culture collection (ATCC), and the assay was carried out as described by Taxvig et al. [43].

All compounds or mixtures were tested in triplicate (0, 0.01, 0.375, 1, 3, 10, and 30 μM) and were tested both in the absence or presence of 0.22 nM T3 (T3-EC50) to test for agonistic and antagonistic potency. Control wells contained cells and test medium with the same amount of DMSO [0.1%] as the exposed cells. After incubation for 96 h, cell growth was measured using the dye resazurine [44]. 100 μl, of a 0.005 mg/ml resazurine solution in PBS, was added to each well, and plates were incubated 3 h at 37 °C, protected from light. Fluorescence was measured (excitation wavelength 560 nm/emission. 590 nm) on a Wallac Victor 1420 multilabel counter (PerkinElmer Life Sciences).

2.8.3. Steroid synthesis in the H295R cell line

The pesticides were investigated for effects on the synthesis of estradiol, progesterone, and testosterone in the human adrenocortical carcinoma cell line H295R (ATCC, CRL-2128) as described previously [45]. In brief, cells were seeded at a density of 3×10^5 cells/well in 24-well culture plates (Costar 3524, Corning, NY, USA) with DMEM/F12 medium (Gibco, Paisley, UK) supplemented with 2.0% Nu-serum (BD Sciences Denmark) along with 1% ITS + premix (containing 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml BSA and 5.35 μg/ml linoic acid; BDSciences Denmark) and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2/air.

The pesticides were added (1 ml) to the cells in triplicates at six concentrations. Control wells contained the same amount of DMSO (0.1%) as exposed cells. After 48 h incubation the medium was removed and stored at −20 °C until analysis. Hormones were measured using commercial hormone kits from Wallac Delfia (PerkinElmerDenmark, Hvidovre, Denmark). Cytotoxicity was evaluated by means of the MTT assay as described by Mosman [46], measuring the absorbance at 570 nm after 1.5–2 h incubation on a Wallac multilabel 1420 counter.

3. Results

3.1. Pregnancy, litter and offspring data

Pregnancy and litter data are shown in Table 2. There were no statistically significant effects on maternal body weight gain from GD 7 to GD 21 or from GD 7 to PD 1 in exposed dams compared to controls. No clinical signs of toxicity were observed in the dams and the number of implantation scars in the uterus, postimplantation and perinatal loss was similar among groups.

Gestation length was significantly increased in the two highest mixture groups (groups 3 and 4) compared to controls ($p < 0.01$ and $p < 0.001$, respectively) and in the group exposed to the highest dose of epoxiconazole ($p < 0.001$), but not in any other groups (Table 2).

Offspring data are shown in Table 3. Pup body weights were unaffected by pesticide exposure at birth and on PD 6, 13 and 22. Nipple retention in male offspring, an indication of anti-androgenic activity, was significantly increased in all mixture groups compared to controls ($p < 0.001$) (Fig. 1). There was also a significant increase

Table 2
Pregnancy and litter data. Data represent group means based on litter means ± SD.

Dams and litters	1: Control	2: Pesti-mix-14.6	3: Pesti-mix-29.2	4: Pesti-mix-43.8	5: Epoxi-3.75	6: Epoxi-15	7: Manz-6.25	8: Manz-25	9: Prchl-8.75	10: Prchl-35	11: Tebu-12.5	12: Tebu-50	13: Procy-12.5	14: Procy-50
No. of dams (litters)	15	17	10 (9)	15 (14)	8	4	5	7	8	4	8	6	7	4
Maternal weight gain GD7–GD21	88.1 ± 13.2	88.6 ± 11.6	86.7 ± 8.6	77.1 ± 12.8	87.3 ± 16.1	84.0 ± 21.1	87.4 ± 5.7	84.1 ± 11.4	85.9 ± 9.6	85.0 ± 5.0	85.4 ± 15.6	82.5 ± 10.2	86.6 ± 10.0	85.0 ± 3.7
Maternal weight gain GD7–PD1	18.5 ± 9.8	17.4 ± 9.5	10.7 ± 8.3	14.6 ± 17.7	22.9 ± 10.2	20.8 ± 9.4	15.4 ± 7.3	13.3 ± 6.5	14.8 ± 4.8	8.8 ± 10.4	11.0 ± 9.6	17.3 ± 15.8	11.6 ± 8.2	6.3 ± 5.5
Body weight gain PD1–13	33.0 ± 9.5	35.5 ± 9.2	31.2 ± 9.3	22.9 ± 19.0	30.9 ± 9.7	33.5 ± 4.0	34.8 ± 8.6	37.3 ± 3.3	34.7 ± 6.1	36.0 ± 8.4	44.8 ± 9.2	33.7 ± 4.2	39.1 ± 6.5	41.8 ± 11.4
Gestation length (days)	23.0 ± 0.0	23.0 ± 0.0	23.5 ± 0.5**	23.6 ± 0.5*	23.1 ± 0.4	23.8 ± 0.5*	23.0 ± 0.0	23.0 ± 0.0	23.2 ± 0.4	22.8 ± 0.5	23.0 ± 0.0	23.1 ± 0.2	23.0 ± 0.0	23.0 ± 0.0
% Post-implantation loss	11.0 ± 24.9	5.4 ± 6.9	6.4 ± 9.9	16.7 ± 25.9	11.7 ± 11.7	4.0 ± 4.6	0.0 ± 0.0	2.3 ± 4.0	3.7 ± 6.0	1.8 ± 3.6	7.9 ± 10.5	5.5 ± 6.5	13.4 ± 35.1	0.0 ± 0.0
% Perinatal loss	14.9 ± 25.2	7.3 ± 8.6	23.4 ± 28.3	23.3 ± 28.6	13.2 ± 12.6	10.3 ± 15.8	1.8 ± 4.1	3.3 ± 4.2	5.4 ± 7.0	1.8 ± 3.6	8.6 ± 12.3	9.5 ± 5.7	17.3 ± 36.9	1.9 ± 3.8
Born alive per. litter	11.1 ± 2.3	11.9 ± 2.5	11.6 ± 2.4	9.8 ± 2.0	10.4 ± 1.8	8.8 ± 3.7	12.2 ± 1.2	12.9 ± 1.7	11.7 ± 1.8	12.0 ± 1.7	11,8 ± 1.9	11.5 ± 2.5	10.8 ± 3.0	12.8 ± 2.4
% Postnatal death	4.4 ± 9.4	2.1 ± 4.3	17.4 ± 30.8	8.9 ± 18.1	1.8 ± 5.1	6.8 ± 13.6	1.8 ± 4.1	1.0 ± 2.7	1.7 ± 5.1	0.0 ± .0	1.0 ± 2.9	4.1 ± 6.6	2.0 ± 5.4	1.9 ± 3.9
% Males	48.4 ± 18.8	47.4 ± 17.5	52.4 ± 13.5	51.6 ± 16.3	44.9 ± 28.1	36.0 ± 29.6	49.8 ± 13.7	45,4 ± 12.9	40.2 ± 11.9	54.3 ± 12.7	56.2 ± 15.2	47.4 ± 16.8	44.6 ± 19.6	53.6 ± 17.3

Epoxi: epoxiconazole; Manz: mancozeb; Prchl: prochloraz; Tebu: tebuconazole; Procy: procymidon; Perinatal loss: loss from implantation to weaning; Postnatal death: death after birth.
* $p < 0.05$.
** $p < 0.01$.

Table 3
Offspring data. Data represent group means based on litter means ± SD. Mix = mixture.

Offspring (data from viable litters)	1: Control	2: pestimix-14.6	3: pesti-mix-29.2	4: Pestimix-43.8	5: Epoxi3.75	6: Epoxi15	7: Manz-6.25	8: Manz-25	9: Prchl-8.75	10: Prchl-35	11: Tebu-12.5	12: Tebu-50	13: Procy-12.5	14: Procy-50
Male birth weight (g)	6.5 ± 0.4	6.1 ± 0.4	6.2 ± 0.4	6.4 ± 0.5	6.5 ± 0.2	6.6 ± 0.6	6.2 ± 0.1	6.2 ± 0.2	6.4 ± 0.4	6.3 ± 0.4	6.3 ± 0.4	6.1 ± 0.4	6.2 ± 0.3	6.4 ± 0.3
Female birth weight (g)	6.1 ± 0.4	5.9 ± 0.4	5.9 ± 0.5	6.1 ± 0.6	6.1 ± 0.3	6.3 ± 0.6	6.1 ± 0.2	5.9 ± 0.1	6.0 ± 0.4	6.0 ± 0.4	6.0 ± 0.3	5.9 ± 0.2	6.1 ± 0.2	6.1 ± 0.3
Male AGD (units)	24.5 ± 1.1	23.4 ± 1.2	24.1 ± 1.0	24.0 ± 1.2	25.7 ± 0.9***	23.9 ± 1.3	23.9 ± 1.0	24.0 ± 0.9	24.8 ± 0.6	24.6 ± 0.7	24.7 ± 1.3	24.6 ± 0.8	22.8 ± 0.9**	22.0 ± 0.7***
Male AGD/cubic root bw (AGDI)	13.1 ± 0.5	12.8 ± 0.7	13.1 ± 0.5	12.9 ± 0.4	13.8 ± 0.4**	12.7 ± 1.0	13.0 ± 0.6	13.1 ± 0.4	13.4 ± 0.4	13.4 ± 0.4	13.4 ± 0.7	13.5 ± 0.4	12.4 ± 0.4**	11.9 ± 0.3***
Female AGD (units)	13.6 ± 0.6	13.7 ± 0.8	14.1 ± 0.5	15.0 ± 0.8***	14.9 ± 1.0**	14.3 ± 0.9	14.2 ± 0.9	13.5 ± 0.5	14.1 ± 0.8	14.5 ± 0.4*	14.4 ± 1.0	14.5 ± 0.5*	13.4 ± 0.4	13.3 ± 0.2
Fem. AGD/cubic root bw (AGDI)	7.4 ± 0.3	7.6 ± 0.5	7.8 ± 0.3*	8.2 ± 0.3***	8.1 ± 0.6**	7.8 ± 0.7	7.8 ± 0.5	7.5 ± 0.2	7.8 ± 0.5*	8.0 ± 0.2**	7.9 ± 0.6*	8.0 ± 0.2**	7.3 ± 0.2	7.3 ± 0.2
No. Areolas males[a]	0.0 ± 0.0	1.9 ± 1.6***	3.3 ± 0.9****	5.3 ± 1.1***	0.1 ± 0.2	0.5 ± 1.0	0.1 ± 0.1	0.6 ± 0.6*	0.3 ± 0.2	1.7 ± 1.2***	0.5 ± 0.8	1.6 ± 0.4***	2.8 ± 1.2***	6.0 ± 2.0***
No. Areolas. females	12.0 ± 0.4	13.4 ± 3.9	12.4 ± 0.4	12.5 ± 0.4	12.4 ± 0.3	12.8 ± 0.5	12.2 ± 0.1	12.4 ± 0.1	12.3 ± 0.2	12.1 ± 0.2	12.5 ± 0.6	12.2 ± 0.2	12.2 ± 0.2	12.4 ± 0.4
Male body weight PD 6 (g)	12.9 ± 1.3	12.2 ± 1.3	12.0 ± 1.3	12.5 ± 1.5	13.1 ± 0.9	13.2 ± 1.0	12.3 ± 0.7	11.8 ± 0.4	12.7 ± 1.2	12.9 ± 1.7	12.1 ± 1.7	12.3 ± 1.2	12.4 ± 0.7	12.2 ± 1.4
Female body weight PD 6 (g)	12.5 ± 1.2	11.9 ± 1.4	11.6 ± 1.4	11.9 ± 1.3	12.8 ± 1.4	12.2 ± 1.0	12.1 ± 0.6	11.4 ± 0.5	12.1 ± 1.2	12.2 ± 1.7	11.9 ± 1.7	12.1 ± 1.2	12.2 ± 0.7	12.0 ± 1.1
Male body weight PD 13 (g)	26.1 ± 4.1	24.0 ± 2.7	25.1 ± 2.9	26.7 ± 3.9	25.9 ± 3.0	28.0 ± 2.4	24.6 ± 2.4	23.0 ± 1.0	25.6 ± 1.5	24.9 ± 2.7	24.2 ± 2.7	24.8 ± 2.0	24.9 ± 1.0	24.3 ± 2.9
Fem. body weight PD 13 (g)	25.4 ± 3.9	23.7 ± 3.0	24.4 ± 2.8	24.3 ± 4.6	26.0 ± 4.1	26.5 ± 2.2	24.4 ± 2.5	22.7 ± 1.4	24.6 ± 1.8	24.0 ± 2.7	23.9 ± 2.1	24.4 ± 2.4	24.5 ± 1.4	23.6 ± 2.8
Male body weight PD 22 (g)	45.6 ± 6.7	41.5 ± 4.4	44.0 ± 5.6	46.8 ± 7.0	43.3 ± 4.6	46.8 ± 3.5	42.9 ± 4.2	40.4 ± 1.6	43.2 ± 3.3	42.4 ± 5.0	43.0 ± 4.6	43.8 ± 4.5	42.5 ± 1.3	43.0 ± 4.4
Fem. body weight PD 22 (g)	45.2 ± 6.5	41.1 ± 4.2	41.9 ± 3.9	44.8 ± 5.8	46.3 ± 7.7	46.7 ± 3.2	42.6 ± 3.5	40.3 ± 2.2	42.3 ± 4.0	41.3 ± 4.9	42.7 ± 3.4	42.8 ± 4.5	42.4 ± 1.3	41.3 ± 4.9

Epoxi: epoxiconazole; Manz: mancozeb; Prchl: prochloraz; Tebu: tebuconazole; Procy; procymidon.
[a] The number of nipples in the control group males was zero and it was therefore necessary to put in 1 nipple in 3 pups from the control group to perform the statistical model.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

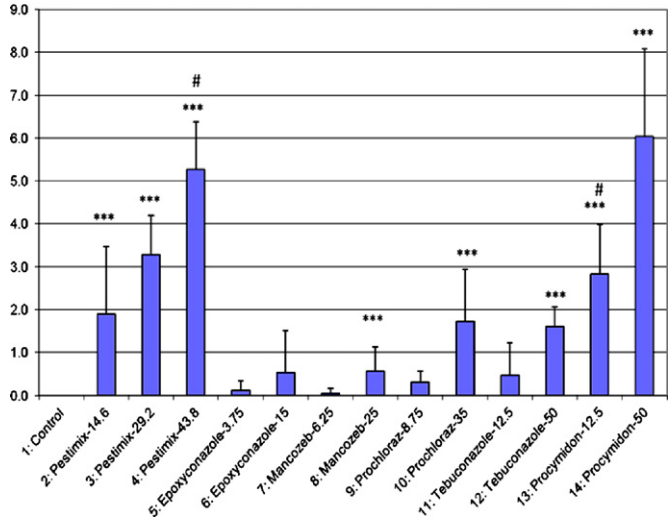

Fig. 1. Effects of single and combined exposure to procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb on nipple retention in male pups PD 13. Results shown are group means ± standard deviation. See Table 3 for more details. The number of nipples in female controls is normally 12. ***p-Values are <0.001. #The difference between Pestimix-43.8 (group 4) and procymidone-12.5 (group13) is statistically significant ($p < 0.001$).

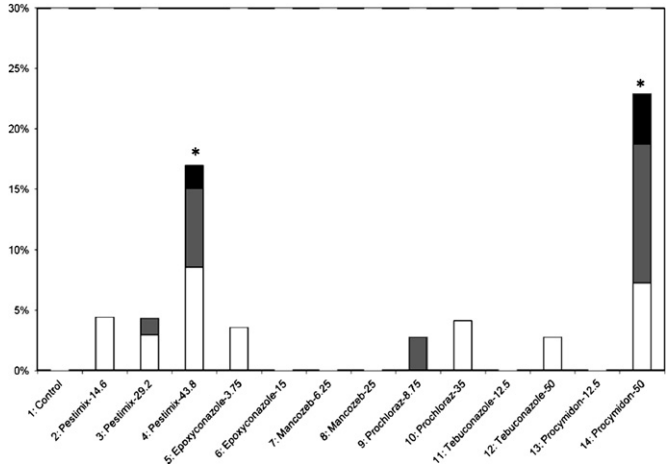

Fig. 2. Genital malformations in male offspring. Results shown are the group mean of the % of the male offspring within each litter given score 1 (white), score 2 (gray) or score 3 (black). The rest of the animals, *i.e.* up to 100%, within each group were given score 0. The incidence of genital malformations is significantly increased ($p = 0.017$) in the highest mixture group (Pestimix-43.8, group 4) and the high dose of procymidone (group 14) ($p = 0.035$).

in the groups exposed to the high dose of mancozeb ($p < 0.05$), prochloraz ($p < 0.001$), tebuconazole ($p < 0.001$) and both doses of procymidone ($p < 0.001$) (Fig. 1). The magnitude of the effects in the highest mixture group was significantly higher than the effect induced by the low dose of procymidone ($p < 0.001$), although the procymidone dose was the same in the two groups.

In the male offspring, no statistically significant changes on anogenital distance (AGD) were seen in the mixture groups. The data for the single chemical exposures showed that the low dose of epoxiconazole induced longer AGD in male pups compared to controls ($p < 0.01$), whereas the high dose had no effect. Both doses of procymidone induced shorter male AGD ($p < 0.01$, and $p < 0.001$, respectively). In the female offspring, a dose-dependent increase in anogenital distance was seen, with significantly longer AGDI, in the two highest mixture groups compared to controls ($p < 0.05$ and $p < 0.001$, respectively). A similar effect was seen in animals exposed to both doses of prochloraz ($p < 0.05$ and $p < 0.01$) and tebuconazole ($p < 0.05$ and $p = 0.001$) and in the group exposed to the low dose of epoxiconazol ($p < 0.01$) but not in female pups exposed to a four times higher dose of epoxiconazole (Table 3).

3.2. Genital malformations in male offspring

The results of the scoring of external genital malformations in male offspring sacrificed on PD 16 and 22 indicated increased number and severity of genital malformation in the highest mixture group and the highest dose of procymidone, but the differences were not statistically significant compared to controls shown in Supplemental Material Tables X2). No animals had increased gubernacular length as an indication of cryptorchidism.

The rest of the male animals were scored for external genital malformations alive shortly after sexual maturation (around PD 50) and the results were combined for all time points (see Fig. 2 and Table 4). The combined results showed significantly increased number and severity of external genital malformation in the highest mixture group ($p = 0.006$) and the highest dose of procymidone ($p = 0.035$), compared to controls. The group mean of the percentage of animals within each litter with external genital malformations was markedly higher in the highest mixture group than in any of the groups dosed with the individual pesticides alone at the dose included in the mixture (around 17% compared to 0–4%, Fig. 2). The individual pesticides alone caused no statistically significant effect at the dose levels included in the mixture.

External malformations related to the reproductive system and seminal vesicles were observed in a few of the nine months old animals (Supplemental Material Table X3). Cryptorchidism and alopecia, in the perineal area, were not observed in any of the adult males. Two males in the highest mixture group had penile malformation: one had both a severe cleft penis and hypospadia with urethral opening at the basis of the penis (score 3), the other had a cleft phallus (score 1). A third male in the highest mixture group had a malformed seminal vesicle. One male in each of the groups dosed with mancozeb showed malformation of the seminal vesicle. In the group dosed with the highest level of procymidone two animals had cleft phallus (scores 2 and 3, respectively), and a third animal had malformed seminal vesicle. However, none of the observed effects were significantly different from control animals.

3.3. Mixture modeling – gestation length

General linear model (GLM) fits for the five pesticides are illustrated in Supplemental Material Fig. X1. The effect on gestation length of mancozeb and procymidone was not statistically significant ($p = 0.25$ for both pesticides). Therefore, mancozeb and procymidone were not included in the predictions for the mixture. A fit of the observed pesticide mixture was performed similar to the single pesticide observations. In addition, predictions were performed based on the models for the single pesticides and the dose-additivity and independent addition formulas. The results are shown in Table 5 and Fig. 3A and B. At high doses, there was a significant difference between the observed and predicted mixture effects, and both of the models underestimated the mixture effects. However, the underestimation was generally smaller for the dose-additivity prediction than the independent action prediction. The dose-addition prediction at the lower doses gave a good prediction of the observed effects. In Supplemental Material Table X4 the effect doses for increased gestation length for individual pesticides are summarized. The overall results were the same when winsorization was used (data not shown).

Table 4
External genital malformations in male pups scored on PD 16 or 22 at sectioning or *in vivo* on PD 50. Values show the number of animals with score 0 (normal), score 1 (slight), score 2 (moderate), or score 3 (severe).

Dose group	No. animals (litters)	Score 0	Score 1	Score 2	Score 3	Score 1, 2 or 3	Statistics
1: Control	71 (15)	71 (15)					
2: Pestimix-14.6	96 (17)	92 (17)	4 (4)			4 (4)	NS
3: Pestimix-29.2	53 (9)	50 (9)	2 (2)	1 (1)		3 (2)	NS
4: Pestimix-43.8	67 (14)	56 (14)	4 (3)	5 (4)	2 (2)	11 (6)*	$p = 0.006$
5: Epoxi-3.75	38 (7)	36 (7)	2 (1)				
6: Epoxi-15	11 (4)	11 (4)					
7: Manz- 6.25	31 (5)	31 (5)					
8: Manz-25	39 (7)	39 (7)					
9: Prchl-8.75	44 (9)	43 (9)	0	1 (1)		1 (1)	NS
10: Prchl-35	26 (4)	25 (4)	1 (1)			1 (1)	NS
11: Tebu-12.5	48 (8)	48 (8)					
12: Tebu-50	27 (6)	26 (6)	1 (1)			1 (1)	NS
13: Procy-12.5	38 (7)	38 (7)					
14: Procy-50	25 (4)	19 (4)	2 (2)	3 (2)	1 (1)	6 (2)*	$p = 0.035$

Values in parentheses are the number of litters with offspring having the indicated score. Epoxi: epoxiconazole; manz: mancozeb; Prchl: prochloraz; Tebu: tebuconazole; Procy: procymidon.
* $p < 0.05$.

Table 5
Observed and predicted mixture effect doses for increased gestation length (GL). Results shown are mean dose [95% confidence interval].

Relative GL	10%	25%	50%	75%	90%
GL (rounded)	23.2	23.5	24.0	24.5	24.8
Observed[a]	18.0 [3.4–31.0]	46.5 [37.5–57.5]	75.5 [64.5–93.5]	104.0 [85.0–127.0]	133.0 [101.5–159.0]
Dose-additivity	9.49 [8.50–10.5]	64.0 [61.6–66.5]	135.3 [132.8–137.9]	197.8 [195.1–200.5]	256.2 [253.2–259.3]
Independent action	0.5 [0.14–0.86]	0.5 [0–4.05]	173.0 [167.4–178.6]	316.5 [310.5–322.5]	439.5 [433.0–446.0]

[a] Based on the curve fit shown in Fig. 3A.

3.4. Mixture modeling – nipple retention in male offspring

In Supplemental Material Fig. X1 the nipple retention results for the single chemicals are shown with the estimated model and ±2 standard errors. No statistically significant effect on NR by mancozeb was observed and the effect was therefore set to zero in the predictions

The fitted model of the observed results and the dose-additivity predictions are illustrated in Fig. 4A and B. Furthermore, the 95% confidence intervals and the estimates of observed data, the dose-additivity predictions, and the independent actions predictions are summarized in Table 6. The results show that for the low doses, the confidence intervals overlapped and there were no apparent statistical differences between the observed dose effects and the dose-additivity predictions. However, at the high doses no overlap in the confidence intervals between the observed dose effects and those predicted by dose-additivity was found. In fact, the dose-additivity formula underestimated the effects for the high doses of the mixture. The independent action predictions were seen to likewise underestimate the effects for high doses, and overestimated the effect at low doses. Furthermore, the independent actions model gave poorer estimates in relation to the observed effects, in comparison to the dose-additivity model. In Supplemental Material Table X5 the effect doses for increased nipple retention in male offspring for individual pesticides are summarized. By using winsorization similar results were obtained (data not shown).

3.5. Mixture modeling – AGDI in female offspring

As there was a significant effect of year on AGDI in female offspring, transformed AGDI observations were used in the analysis. The same model and fitting method (GEE) as for NR was used for effects on AGDI. However, only prochloraz could, based on this, be assumed to have a statistically significant effect on AGDI (data not shown). The relatively few data available for epoxiconazole and tebuconazole suggest that accepting the null-hypothesis of no effect from these pesticides without further examination is arguable.

The fitted model of the observed results is illustrated in Fig. 5A. The results in Table 7 and Fig. 5B show that both independent action and dose-additivity predictions highly underestimated the effect of the pesticide mixture. So, although no statistical effect was seen for epoxiconazole, mancozeb, tebuconazole and procymidone individually, a marked contribution to the effect of the mixture was evident. In Supplemental Material Table X6, the effect doses for longer AGDI in female offspring for prochloraz are shown. Using winsorization, the estimated effect doses were slightly higher for the low effect doses and slightly lower for the high effect doses (data not shown). However, the overall picture was the same.

3.6. In vitro studies

All pesticides except for mancozeb exhibited AR antagonism *in vitro* (Fig. 6).The lowest observed effect levels (LOEC) and cytotoxic concentrations are shown in Supplemental Material Table X7. The ranking of potencies for AR antagonism was: mixture ~ procymidone > prochloraz ~ epoxiconazole > tebuconazole. As the content of procymidone in the mixture constitutes only approximately 1/3 of the total mixture dose, these results indicate combination effects on AR antagonism *in vitro*.

In the H295R steroid synthesis assay, epoxiconazole, prochloraz, tebuconazole, and the mixture were shown to reduce testosterone levels in the cells, whereas mancozeb and procymidone had no effect (Fig. 7). Epoxiconazole, prochloraz, tebuconazole and the mixture also reduced estradiol levels (Fig. 7). Generally the pesticides and the mixture caused an increase in progesterone levels, although for the mixture and for procymidone the effect was only seen at the highest concentrations tested (Fig. 7). In general the effects of prochloraz was most pronounced on all three hormones.

Triiodothyronine (T_3) induced dose-dependent proliferation of the GH3 cells in the T-screen assay as expected (data not shown). No agonistic effect was observed for the mixture (Supplemental

Table 6
Observed and predicted mixture effect doses for the increased nipple retention (NR) in male offspring. Results shown are mean dose [95% confidence interval].

Relative NR	10%	25%	50%	75%	90%
NR (rounded)	1	3	6	9	11
Observed[a]	2.3 [0.01–4.9]	27.0 [25.5–28.5]	51.5 [49.5–53.5]	76.0 [72.5–79.5]	100.5 [95.0–105.5]
Dose-additivity	0.021 [0.020–0.022]	18.85 [17.6–20.1]	98.39 [97.3–99.5]	153.53 [151.9–155.1]	206.45 [204.3–208.5]
Independent action	0.01 [0.01–0.01]	0.01 [0.01–0.01]	41.5 [39.8–43.1]	184.0 [182.5–185.5]	289.0 [287.4–290.6]

[a] Based on the curve fit shown in Fig. 4A.

Table 7
Observed and predicted mixture effect doses for increased anogenital index (AGDI) in female offspring. Results shown are mean dose [95% confidence interval].

Relative AGDI	10%	25%	50%	75%	90%
AGDI (rounded)	8.04	8.89	10.30	11.72	12.56
Observed[a]	38.0 [34.0–42.0]	104.0 [92.0–124.0]	170.0 [147.0–202.5]	236.0 [199.0–275.0]	302.0 [245.0–346.5]
Dose-additivity	224.0 [217.8–230.3]	720.6 [709.6–731.6]	1217 [1200–1234]	1714 [1691–1737]	2210 [2180–2239]
Independent action	209.5 [202.9–216.1]	694.5 [687.9–701.1]	1228 [1211–1245]	1750 [1726–1773]	2255 [2226–2285]

[a] Based on the curve fit shown in Fig. 5A.

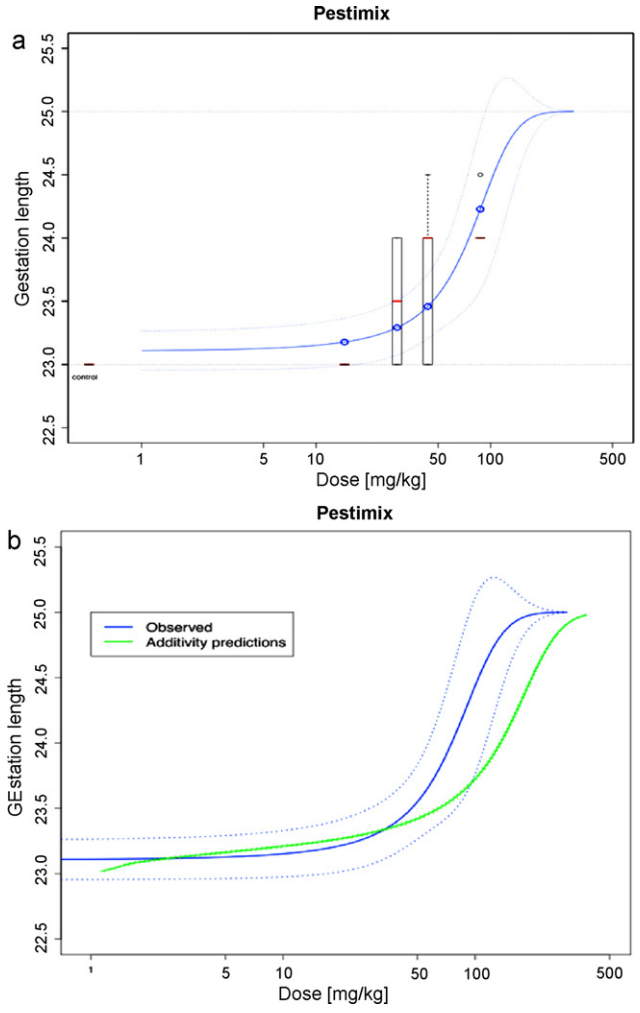

Fig. 3. (A) GLM fit of mixture effect on gestation length (GL). The observations are shown as box plots, red marks = median of observations at a given dose. (B) The observed and predicted mixture effects on gestation length GL. The dose-additivity prediction of the mixture effect doses are in green and the observed curve is in blue. In dotted lines ±2 standard errors are illustrated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

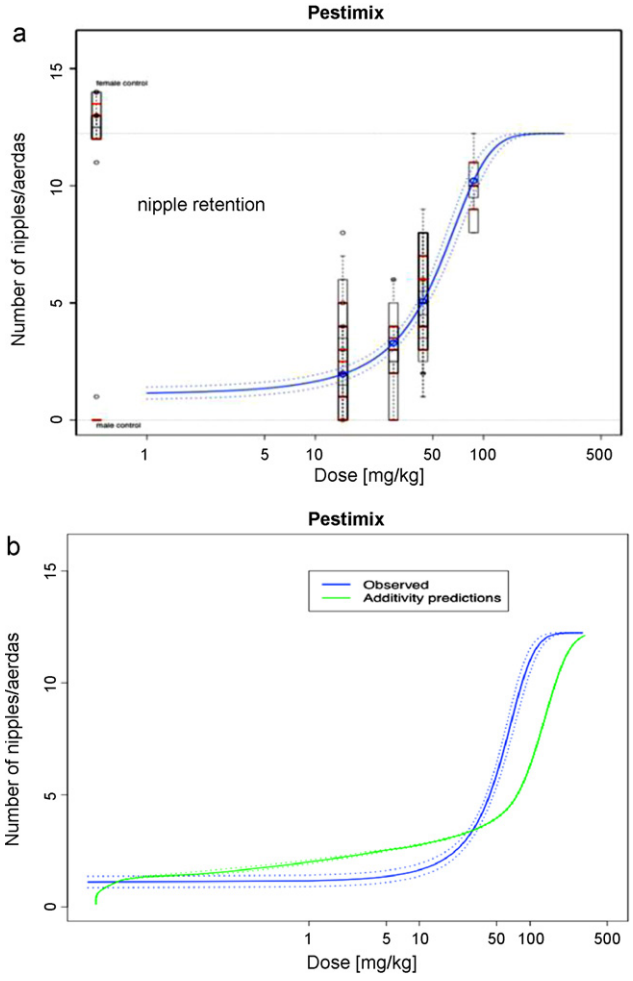

Fig. 4. (A) GEE fit of mixture effects on nipple retention in male offspring ±2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter medians. (B) The observed and predicted mixture effect on nipple retention in male offspring. The dose-additivity predictions of the mixture effect doses are in green and the observed curve is in blue. In dotted lines ±2 standard errors are illustrated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

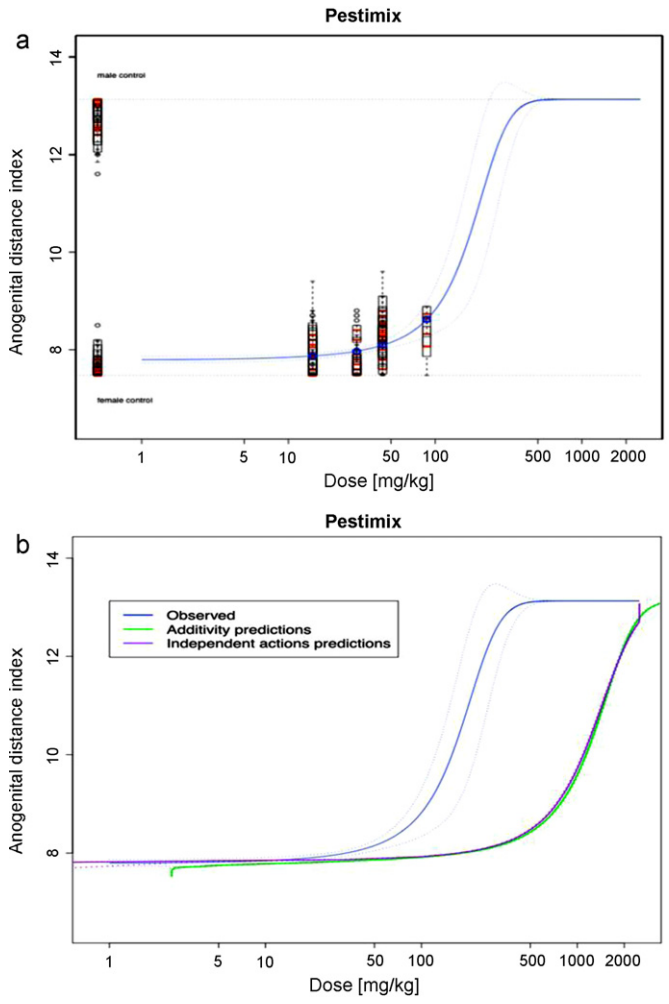

Fig. 5. (A) GEE fit of mixture effects on anogenital index (AGDI) in female offspring ±2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter medians. (B) The observed and predicted mixture doses for effects on anogenital index in female offspring. The dose-additivity predictions are in green, the independent action predictions are in purple. And the observed curve in blue. In dotted lines ±2 standard errors are illustrated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

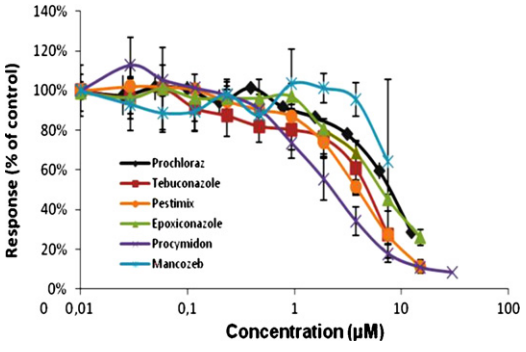

Fig. 6. Androgen receptor antagonism of single pesticides and the mixture (pestimix) *in vitro*.

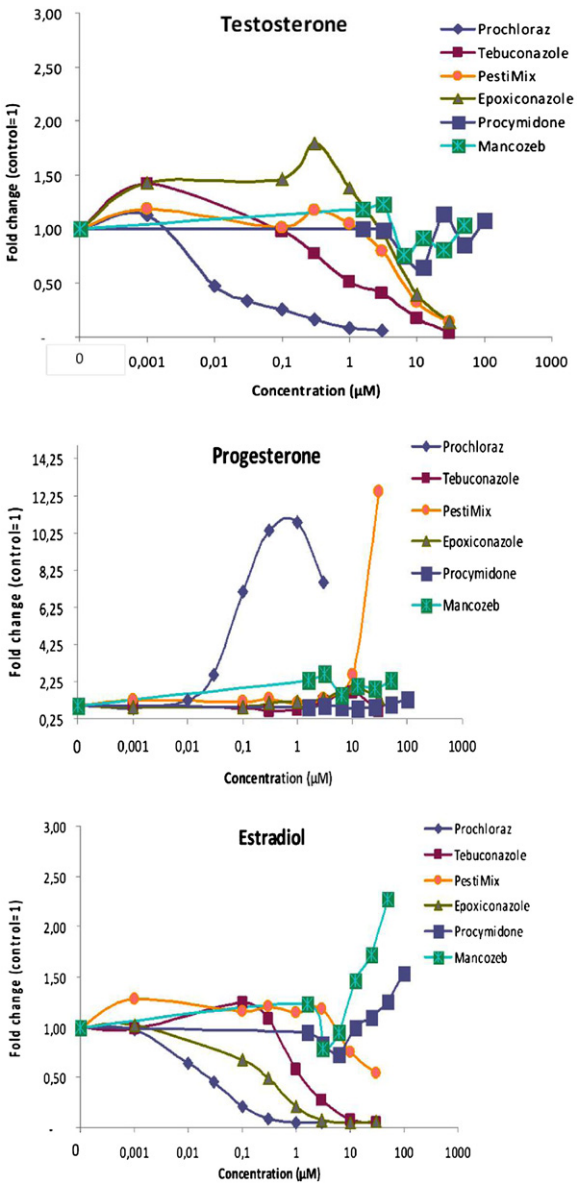

Fig. 7. Changes in levels of testosterone (top) estradiol (middle) and progesterone (bottom) in the H295 steroid synthesis assay after exposure to increasing concentrations of the 5 pesticides singly or in combination.

Material Fig. X3a). A weak antagonistic effect of the mixture was observed at concentrations at and above 3.13 µM (Supplemental Material Fig. X3b). A summary of all *in vitro* results is shown in Supplemental Material Table X7.

4. Discussion

This study generally aimed to explore the hypothesis that combined developmental exposure to endocrine disrupting pesticides could lead to adverse developmental toxicity effects at dose levels below NOAELs for the single pesticides, and to investigate if dose-additivity modeling of the expected mixture effects could give useful estimates of the observed mixture effects.

4.1. Developmental mixture study and mixture modeling

There were no statistically significant effects of pesticide exposure on maternal body weight gain or on pup body weights at birth or in the postnatal period. For the mixture, this was consistent

with the results from a previous range-finding study [23] in which maternal and pup body weight gains were only affected at much higher doses of the mixture.

Several of the investigated endocrine and reproductive endpoints were affected by the combination of the five pesticides, and the effects were seen at dose levels at which the individual pesticides caused no, or only minor, effects (Table 8). Gestation length was significantly increased in the two highest mixture groups and in the group exposed to the highest dose of epoxiconazole, but not in any other group. The mixture doses consisted of each of the pesticides at 17% or 25% of their NOAEL for effect on gestation length based on results of previous studies in our laboratory. As the highest dose of epoxiconazole given alone was four times higher than the dose included in the highest mixture group, these results showed a combination effect of the pesticides at dose levels where the individual pesticides caused no effects. Increased gestation length of very similar magnitude was also observed in a similarly exposed mixture group in the range-finding study [23]. In that study, combined exposure at higher doses induced severe effects manifested as dystochia (impaired parturition) and high perinatal pup mortality [23].

To our knowledge, this is the first study to describe mixture effects on gestation length. The mixture effect is probably due to the presence of the three azole fungicides in the mixture, epoxiconazole, prochloraz and tebuconazole, which have previously been shown to elicit such effects on gestation length [18,47,48]. Prolongation of the gestation period may possibly be due to an increase in progesterone in the dams as suggested for epoxiconazole, prochloraz and tebuconazole [18,20]. It has previously been shown that all three azoles are able to induce progesterone levels *in vitro* [41] and this may be the cause of the increased gestation length *in vivo* as a decline in progesterone levels in the dams is a prerequisite for on-time delivery in rats [49].

The dystochia and pup mortality seen in the range finding studies have previously been observed as common effects for several azole fungicides [18,47,50,51]. The mode of action for these endpoints may be increased progesterone levels as well, and if this is the case, then these azole fungicides should be considered as similarly acting. However, further data are needed to make firm conclusions. Neither mancozeb nor procymidone have previously been shown to affect gestation length or perinatal survival at the doses studied [7,21].

For the endpoint gestation length, application of the dose-addition model at low doses resulted in a good prediction of the observed mixture effects. Both dose-additivity and independent action model predictions underestimated the mixture effects at high doses. However, the underestimation was generally smaller for the dose-additivity prediction than the independent action prediction. Our hypothesis is that the underestimation at high doses may be due to toxicokinetic interactions as *e.g.* saturation of metabolism. We have preliminary data to support this view as higher pup blood levels of some of the pesticides was found after mixture exposure than after exposure to the pesticides alone [25]. This is the first study to show mixture effects on the complex endpoint gestation length, and also the first study to show that it is possible to predict mixture effects using dose-additivity for this more 'functional' and biologically relevant endpoint as well.

Nipple retention in male offspring was significantly increased in all mixture groups. For the highest mixture group, the results were consistent with the results from the range-finding study [23]. Nipple retention was also increased at the highest dose of mancozeb, prochloraz and tebuconazole and at both doses of procymidone. However, the effect of the high dose of mancozeb (25 mg/kg) was numerically quite small. As an earlier study of mancozeb including larger groups and higher dose levels (up to 100 mg/kg) did not show any effect on nipple retention [21], the significant effect in

Table 8
Summary of effects found in dams and male rat pups exposed to epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu) or procymidone (Procy), or a mixture of these pesticides (Pestimix) from GD 7 to PD 16.

Endpoint	Epoxi 3.75 mg/kg	Manz 6.25 mg/kg	Prchl 8.75 mg/kg	Tebu 12.5 mg/kg	PROcy 12.5 mg/kg	Pestimix 43.8 mg/kg	Dose-additivity ?	Joint effect compared to effect of single chemicals at Mix-25%
Gestation length, days	23.1	23.0	23.2	23.0	23.0	**23.6**	Yes, slight synergy at high doses	Marked joint effect; no significant effect of single chemicals
Nipple retention, PND 13, number	0.1	0.1	0.3	0.5	**2.8**	**5.3**	Yes, slight synergy at high doses	Marked joint effect; smaller significant effect of procymidone
Genital malformations, %								
Score 1	3.6	0.0	0.0	0.0	0.0	8.6	n.a.	Marked joint effect; no significant effect of single chemicals
Score 2	0.0	0.0	2.8	0.0	0.0	6.5		
Score 3	0.0	0.0	0.0	0.0	0.0	1.9		
Total	3.6	0.0	2.8	0.0	0.0	**17.0**		

n.a.: not analyzed. Values are expressed as percent of control values for relative organ weights and as increase in number for nipple retention (control value = 0.0). Values for genital malformations of external male reproductive organs are shown as group mean of % offspring within each litter having score 1, 2 or 3 and the sum (control value = 0.0%). Statistically significant effects compared to controls are indicated by bold letters.

the present study is likely to be a random finding. For the other pesticides, similar effects have to some extent been seen for the individual pesticides [6,16,18,20,26,47,51–53]. No statistically significant effects on nipple retention of mancozeb, prochloraz and tebuconazole were found at the low dose level, whereas procymidone caused a significant effect (Table 8). Results on nipple retention indicate mixture effects, since the magnitude of the effect in the highest mixture group was significantly higher than the effect induced by procymidone alone at the dose level included in the highest mixture. Mixture effects for nipple retention have earlier been shown after exposure to other mixtures of both similarly and dissimilarly acting anti-androgens in several rat studies [6,26,54].

The mixture effect prediction based on dose-additivity was in agreement with the observed effects at low doses, as there were no statistically significant differences between the predicted and the observed effect doses. However, dose-additivity generally underestimated the effects for the high doses of the mixture. The independent action predictions resulted in even stronger underestimations of the effects for high doses, and overestimations of the effect for the low doses. It has been reported previously that dose-additivity gives a good prediction of mixture effects of nipple retention with both similarly and dissimilarly acting anti-androgens [6,26,54].

In male offspring, no statistically significant effects on anogenital distance (AGD) were seen in the mixture groups. At the dose levels included in the mixture, epoxiconazole induced longer AGD, whereas procymidone induced shorter AGD. Consequently, the lack of effect in the mixture group might be due to opposite actions of these compounds.

In the female offspring, significantly longer AGDI, was seen at the two highest mixture doses. A similar effect was seen in female offspring given the low dose levels of epoxiconazole, prochloraz and tebuconazole, *i.e.* at doses equal to those included in the highest mixture dose. No effect was seen in female offspring dosed with a four times higher dose of epoxiconazole and we hypothesize that the finding at the low dose may be a random finding or alternatively, the lack of effect at the high dose may be due to a limited group size. However, the high doses of both prochloraz and tebuconazole did cause increased female AGDI in the present study. The AGD effect caused by the mixture is probably due to the three azole fungicides as earlier studies in our laboratory have shown similar effects for epoxiconazole, prochloraz and tebuconazole [18,53]. The mechanism underlying the increased AGDI in the females may be the increased progesterone levels in the dams causing virilization of the female genitals [55].

Both independent action and dose-additivity predictions highly underestimated the effect of the pesticide mixture on AGDI in females. So, although statistically significant effect was neither seen in the combined analysis of the results in the present study, nor in the historical data for epoxiconazole, mancozeb, tebuconazole and procymidone individually, some of the pesticides seem to have contributed to the effect observed after mixture exposure. The present study showed significant effects of the low doses of epoxiconazole, prochloraz and tebuconazole, whereas no effect on female AGDI was seen for mancozeb and procymidone. Earlier studies in our laboratory have also shown similar effects of epoxiconazole, prochloraz and tebuconazole [18,53]. The effect in the mixture groups was therefore most likely caused by the combined exposure to the three azole fungicides. The finding that mixed exposure to endocrine disrupters can lead to increased female AGD is new and more knowledge on this endpoint is needed.

An increased incidence and severity of malformations in the highest mixture group and the highest dose of procymidone was found. At the same mixture dose, the incidence of genital dysgenesis in the range-finding study [23] was around 40%, whereas the incidence in the present mixture study was around 17%. This difference may be related to the lower number of litters in the groups in the range-finding study and hence greater uncertainty in results. The percentage of offspring with genital malformations was markedly higher in the highest mixture group than in any of the groups dosed with the individual pesticides alone at the dose included in the mixture (Table 8). The individual pesticides alone caused no statistically significant effect at this dose level. As such, these results show severe mixture effects at dose levels where the individual pesticides caused no effect when given alone. Similar results were found in another mixture study of three similarly acting anti-androgens, *i.e.* the AR antagonists flutamide, procymidone and vinclozolin [6]. Modeling was not done for genital malformations, because there were insufficient data for the single pesticides regarding this endpoint.

4.2. In vitro studies

The objective of performing the *in vitro* assays was to evaluate the predictability of *in vitro* methods for estimating *in vivo* developmental effects. The ranking of the potencies for AR antagonism was: mixture ~ procymidone > prochloraz ~ epoxiconazole > tebuconazole. As the content of procymidone in the mixture actually constituted only approximately 1/3 of the total mixture dose, these results indicate combination effects on this endpoint. This is in line with previous observations that chemicals act additively to antagonize AR [56].

Epoxiconazole, prochloraz and tebuconazole and the mixture were all shown to reduce testosterone and estradiol levels and increase progesterone levels in H295R cells. In general the effects of prochloraz was most pronounced on all three hormones. In regards to translating the *in vitro* results into *in vivo* effects in developmental rat studies, the lack of effects of mancozeb on steroid hormone synthesis *in vitro* fit well with the lack of observed anti-androgenic effects of mancozeb *in vivo*. Based on the *in vitro* results, one might also expect to see an effect on the measured hormone levels, particularly on the progesterone levels in the rats. In the present study, progesterone levels were unfortunately only investigated in the offspring at 50 days of age, and here no significant effects were seen [24]. However, results from previous *in vivo* studies with azole fungicides show that pregnant rats dosed with epoxiconazole (15 and 50 mg/kg bw/day) and tebuconazole (50 and 10 mg/kg bw/day) during gestation had a marked increase in progesterone levels in plasma at GD 21 [18].

Concerning the prediction of anti-androgenic effect (*e.g.* nipple retention), we would expect from the *in vitro* studies that procymidone and the three azole fungicides had the potential to induce this effect. Procymidone was the most potent AR antagonist *in vitro* and had also effects on nipple retention. Prochloraz was the most potent testosterone inhibiting chemical *in vitro* and was found to affect nipple retention in the present study and has previously been shown to reduce male AGD *in vivo*, although higher doses than those used in this project were necessary to induce this effect [53]. All taken together, the *in vitro* studies gave good indications of whether to expect *in vivo* anti-androgenic effects and effect on gestation length. Overall, application of predictable *in vitro* assays may contribute to faster and cheaper data generation for evaluation of chemicals and a reduction of the number of experimental animals that have to be used for risk assessment of chemicals.

4.3. Regulatory perspectives

In the present paper mixture effects of endocrine disrupting pesticides have been shown, and as such the results support that there is a need for risk assessment of the cumulative exposure to chemicals. Below the mixture results are discussed in relation to values used in regulatory risk assessment of single chemicals such

Table 9
LOAELs for effect on gestation length (GL) in dams and nipple retention (NR) in male offspring after exposure to the single pesticides alone or in mixture.

Pesticide	Epoxiconazole		Prochloraz		Tebuconazole		Procymidone	
	Single	Mixture	Single	Mixture	Single	Mixture	Single	Mixture
LOAEL-GL	15	2.5	>35	5.8	>50	8.3	–	–
LOAEL-NR	>15.0	1.3	5.8	2.9	8.3	4.2	4.2	4.2

Mancozeb and procymidone are not included for GL, and mancozeb is not included for nipple retention, as the pesticides did not affect these endpoints.

Table 10
ADI and derived no effect level (DNEL) in mg/kg bw/day for nipple retention in male offspring after exposure to the single pesticides alone or in mixture.

	ADI[a]	DNEL single	DNEL mix
Epoxiconazole	0.008	0.050	0.004
Prochloraz	0.010	0.019	0.010
Tebuconazole	0.030	0.028	0.014
Procymidone	0.003	0.014	0.014

DNEL is calculated as LOAEL divided by 300. Mancozeb is not included as this pesticide did not affect nipple retention.

[a] The ADI values for the four pesticides respectively are from EC (2008a), FAO (2004), EC (2008b) and EFSA (2009).

as LOAELs, Derived No Effect Levels (DNELs) and Acceptable Daily Intake (ADI).

The observed LOAELs for the pesticides singly and the doses of the pesticides in the mixture at the mixture LOAEL were compared for the endpoints gestation length and nipple retention (Table 9). This was done only for, the relevant pesticides in the mixture, *i.e.* those that singly caused effects on these endpoints. The main purpose was to estimate whether mixture exposure would lead to lower LOAELs than seen after single chemical exposure. For gestation length, the doses of the single pesticides at the mixture LOAEL were approximately 6 times lower than the LOAELs for the pesticides given alone. This is an important finding, as it further illustrates that mixture exposure leads to lower effect levels. For nipple retention, several of doses of the single pesticides at the mixture LOAEL were also lower than those for single chemical exposure. However, for procymidone, there was no difference between single and mixture LOAELs. Also, the differences between single and mixture LOAEL for prochloraz and tebuconazole was only 2 times, and not 6 times like it was seen for gestation length. It must, however, be kept in mind that the mixture caused effect on nipple retention at the lowest mixture dose studied, *i.e.* the mixture LOAELs may have become lower if lower doses of the mixture had been studied. LOAELs determined experimentally depend on many factors such as the selection of strain or species, the selection of doses levels, sample size, the end points assessed and biological variation. Thus some variability is to be expected among LOAELs and may range from less than twofold up to a factor of 10 [57]. However, the LOAELs in this study are from studies in the same strain of rats, the dose levels were carefully selecting based on the previous results in our laboratory and the same endpoints were assessed. Also, the results were quite reproducible for both the single pesticides and the mixture when comparing the results from our previous studies on the single pesticides, the range-finding study with the mixture and the large mixture study where both mixture doses and doses of the single pesticides were included ([23], Supplemental Material Table X1). Thus, the variation in our study LOAELs are clearly below a factor of 6 substantiating that this factor represents an increase beyond variation within and between studies.

In Table 10, the Acceptable Daily Intakes (ADIs) are compared to the DNELs for effect on nipple retention for each of the pesticides. The DNEL was calculated from the LOAELs shown in Table 9 using the normally used uncertainty factors for deriving ADIs, *i.e.* 10 for extrapolation from animals to humans, 10 for different sensitivity among humans and 3 for extrapolation from an effect level to NOAEL. All of the DNELs for single chemical exposure to the pesticides were higher or similar to the ADIs, reflecting that the current ADI probably protects against the endocrine disrupting effect of the studied pesticides, when the pesticides are evaluated chemical by chemical. The mixture DNELs were, however, in most cases lower than the ADIs. This was especially the case for epoxiconazole and tebuconazole, where the DNELs based on the respective LOAELs were half of the ADI. This indicates that the ADIs are not sufficiently low to protect against the mixture effects of the pesticides studied in this project. An exception was procymidone where the ADI was lower than the mixture DNEL.

A survey of the dietary cumulative intake using the probabilistic approach did not show a reason for concern in relation to mixed exposure of Danish consumers to the five investigated pesticides (Jensen et al. [58]). The risk assessment included an additional uncertainty factor of 3 to allow room for human exposure to other endocrine disrupting chemicals. However, it is somewhat uncertain whether this factor is sufficient for covering the already known endocrine disrupters and especially the thousands of chemicals that are potential endocrine disrupters based on *in vitro* data and QSAR modeling [59,60].

4.4. Conclusions

Combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides caused adverse effects on male sexual development and gestation length and the mixture effects were well predicted by dose-addition at low doses.

The *in vitro* data showed that the AR reporter gene assay and the H295R steroidogenesis assay gave good indications of the observed *in vivo* effects. As humans may be exposed to thousands of chemicals for which we still have no data about endocrine disrupting properties, further *in vitro* studies as well as QSAR modeling are important for progress in this area. *In vitro* data are especially important for decisions on how to group endocrine disrupters for cumulative risk assessment.

Comparisons of the DNELs for the mixture effect on nipple retention to the Acceptable daily intake (ADI) indicate that ADIs are not sufficiently low to protect against the mixture effects of the pesticides shown in this study. Thus, the results imply that risk assessments based on NOAELs for single chemicals likely underestimate the risk and that there is a need for modification of risk assessment procedures for pesticides, in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction. One possibility is to allow each pesticide to use only a fraction of the ADI, where the fraction should depend on the number of relevant endocrine disrupters in the specific exposure scenario. Another possibility is to base the cumulative risk assessment on dose-additivity modeling. Observations of similar type of effect are recommended as the basis for grouping chemicals for cumulative risk assessment as the results of our study support findings from previous studies showing dose-additive mixture effects of endocrine disrupters that induce similar types of effects, but have dissimilar mechanisms of action. Last but not least, it is recommended when considering cumulative

risk assessment to include all kinds of chemicals *e.g.* pesticides, industrial chemicals, environmental contaminants, as endocrine disrupters exist within all of these chemicals classes and humans may be exposed to several of them simultaneously.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.05.090.

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Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides

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ABSTRACT

There is growing concern of permanent damage to the endocrine and nervous systems after developmental exposure to endocrine disrupting chemicals. In this study the permanent reproductive and neurobehavioral effects of combined exposure to five endocrine disrupting pesticides, epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, were examined. Pregnant and lactating rat dams were dosed with a mixture of the five pesticides at three different doses, or with the individual pesticides at one of two doses.

Adverse effects were observed in young and adult male offspring from the group exposed to the highest dose of the mixture. These included reduced prostate and epididymis weights, increased testes weights, altered prostate histopathology, increased density of mammary glands, reduced sperm counts, and decreased spatial learning. As no significant effects were seen following single compound exposure at the doses included in the highest mixture dose, these results indicate cumulative adverse effects of the pesticide mixture.

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1. Introduction

Exposure to endocrine disrupting chemicals (EDCs) during early life may cause long-term health effects, and can influence both the sexual and neurological development of the offspring, even until it reaches maturity or middle age [1–3]. In the Western world, findings of declining human semen quality and a high prevalence of congenital malformations of reproductive organs and hormone-dependent cancers [4–6], as well as a high prevalence of children being diagnosed with ADHD and other neurological disorders [7,8] are causing concern.

Previous research indicates that a wide range of pesticides may act as endocrine disrupters. The azole fungicides prochloraz, tebuconazole and epoxiconazole have been shown to react through several endocrine disrupting mechanisms, and to induce various endocrine disrupting effects [9–15]. Common features for the azole fungicides are that they increase gestational length and affect steroid hormone levels in fetuses and/or dams. In addition, studies indicate that prochloraz may also affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain [11]. Furthermore, it has been shown that procymidone competitively antagonizes binding to the androgen receptor (AR), and consequently affects the reproductive development in male offspring [16,17]. Mancozeb, a fungicide from the dithiocarbamate group, mainly acts via disruption of the thyroid hormone system and is therefore suspected of affecting brain development [18,19].

Although animal studies have shown that some pesticides can disrupt male sexual differentiation during development, the individual pesticides alone have so far not been shown to contribute to adverse human effects at relevant exposure levels. However, initial observations in epidemiological studies [20–22] point in the same direction as what has been seen in laboratory experiments

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with endocrine disrupting chemicals, namely that substantial mixture effects occur even though the individual chemicals are present at low, ineffective doses [23–25]. Cumulative effects can be seen when small and statistically insignificant effects of each compound are added to induce statistically significant effects when these compounds are mixed. These findings have stimulated interest in exploring the consequences of combined exposures to environmentally relevant mixtures of endocrine disrupting pesticides.

Currently, there are no data on the effects of combined developmental exposure to endocrine disrupting pesticides, which have the potential for affecting both reproductive and brain development. It is important to keep in mind that some pesticides may act through both sex- and thyroid hormone related mechanisms. Furthermore thyroid hormone disrupting pesticides may also affect testicular development [26] while anti-androgenic pesticides may disturb the sexually dimorphic development of the brain [11,27]. Consequently, it is relevant to study combined effects of pesticides with such dissimilar modes of action. Therefore, this study aimed at exploring whether combined developmental exposure to endocrine disrupting pesticides at low doses, i.e. doses below NOAEL for the single pesticides, would lead to adverse developmental toxicity effects. In the present paper we report results on thyroid and reproductive organs and behavioral endpoints from pre-pubertal and adult animals that have been exposed pre- and postnatally to a mixture of the five endocrine disrupting pesticides; procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb. Data on maternal endpoints, postnatal development and genital malformation frequencies from this study as well as mathematical modeling of the mixture results and in vitro studies with the same mixture of pesticides are presented in Hass et al. [28].

2. Materials and methods

2.1. Chemicals

Before initiating the study, the mixture ratio and dose levels of the individual pesticides were chosen as presented in Hass et al. [28]. In summary, the mixture ratio for the five pesticides was chosen based on the NOAEL for effects on increased gestation length in dams and perinatal mortality in the offspring. Upon choosing the mixture ratio, two range-finding studies were performed in order to test for toxicity and endocrine disrupting effects of various mixture doses. The first was in non-pregnant animals while the second was in pregnant animals [29].

The 5 pesticides used were procymidone (CAS no. 32809-16-8, purity 99.5), epoxiconazole (CAS no. 106325-08-8, purity 99.0), tebuconazole (CAS no. 107534-96-3, purity 98.5), mancozeb (CAS no. 8018-01-7, purity 76.0) and prochloraz (CAS no. 67747-09-5, purity 98.5). All chemicals were purchased in a technical quality from VWR – Bie & Berntsen, Herlev, Denmark. Corn oil (Sigma–Aldrich, Brøndby, Denmark) was used as vehicle.

2.2. Animals and exposure

The mixture study was performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee. Animals received a complete rodent diet and acidified tap water ad libitum, and were housed under standard conditions with 12 h reverse light–dark cycle with light starting at 9 p.m. and continuing throughout the night until 9 a.m. In this way behavioral testing could be performed during the animals' active period. For further information on housing conditions please consult Hass et al. [28].

The study included 14 groups of animals, and was performed in 4 blocks with a week between each block. The 14 groups were as equally as possible distributed among the 4 blocks, and the animals used were 198 time-mated nulliparous, young adult female Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark). The animals were observed twice daily for signs of toxicity and body weights were recorded daily during the entire dosing period.

On the day after arrival at gestation day (GD) 4, the dams were distributed into groups with similar body weight (bw) distributions. They were given 4 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. Dams were dosed daily by gavage, from GD 7 to pup day (PD) 16. For more detailed information on dosing scheme please consult Hass et al. [28].

In Table 1 the composition of the pesticide mixture, the doses of the pesticides administered individually and in mixture and the number of litters in each group are shown. Four groups of 22 rat dams were given daily oral doses of 0, 14.6 (8.3% of NOAEL), 29.2 (17% of NOAEL) or 43.8 (25% of NOAEL) mg/kg/day of the mixture of the 5 pesticides, whereas ten groups of 10 or 12 time-mated rats were similarly dosed with two doses of the individual pesticides. The lowest dose of each pesticide was similar to the dose included in the highest mixture dose and the highest dose of the single pesticides was 4 times higher, corresponding to 25% of NOAEL and to NOAEL, respectively, for effects on gestation length and perinatal mortality. Due to low pregnancy rate the number of litters in each dose group was unfortunately somewhat lower (Table 1).

In Fig. 1 an overview of the study design is given. Results from offspring sacrificed on PD 16 and after weaning are presented in the present paper, whereas results from dams and the younger pups are presented in Hass et al. [28].

2.3. Sacrifice on PD 16

On PD 16, 1–3 male and 1–3 female pups per litter were randomly selected for autopsy. Pups were weighed, decapitated and trunk blood was collected for hormone analysis. Uterus, ovaries, thyroids and liver were dissected from one female pup per litter. Uterus, ovaries and livers were weighed, whereas the thyroid was excised on the thyroid cartilage in order to obtain optimal histological preservation. Uterus, one ovary, alternately left and right, a section of the liver and the thyroid were fixed in formalin and processed for paraffin embedding.

Testes, epididymides, ventral prostate, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands, liver and thyroids were dissected from one male pup per litter and weighed. One testis per male (alternately left and right) was fixed in Bouin's fixative and processed for paraffin embedding. Epididymides, seminal vesicles and thyroids (cleared from the thyroid cartilage) were fixed in formalin and processed for paraffin embedding.

Histological evaluation was made of testes, thyroids and of those organs in which statistically significant changes in organ weights were seen. One section per organ was stained with hematoxylin and eosin for histological evaluation, and for thyroids only the mixture groups were evaluated.

2.4. Sacrifice on PD 22 and 50

On PD 22, 1–3 male and 1–3 female pups per litter were weaned. Dams were decapitated in CO_2/O_2 anesthesia and the numbers of uterine implantation sites was counted. Trunk blood was collected and used for hormone analysis. The male and female pups which were not to be kept after weaning were decapitated on PD 22 in CO_2/O_2 anesthesia and blood samples were collected for hormone analyses. On PD 50 1–2 males and females per litter from control and mixture groups were decapitated in CO_2/O_2 anesthesia and blood samples were collected for hormonal analyses.

2.5. Mammary glands

From controls and the three mixture groups (groups 1–4), one male and one female per litter at PD 22 and 1–2 males and 1–2 females per litter at PND 50 were used for investigation of effects on mammary gland development. At both ages, the 4th abdominal mammary gland was excised for whole mount preparation, and on PD 50 the contralateral 4th abdominal mammary gland from males and females was excised for histological analysis. Alternately left and right glands were used for each purpose. For histologic examination, mammary glands were fixed in formalin and stained with hematoxylin and eosin. Female mammary glands were evaluated for tubuloalveolar and lobuloalveolar morphology. Male mammary glands were evaluated for secretory material in the ducts and vacuolization of the epithelium in controls and the highest mixture group. The mammary gland whole mounts were fixed in formalin, stained with alum carmine, dehydrated and mounted. The mammary glands were scanned on a flatbed scanner and outline area, longitudinal growth, and transverse growth were measured using Image Pro Express (Media Cybernetics). The density was scored on a scale from 1 to 5 (with 5 representing most dense mammary glands) with appropriate scoring criteria according to age and gender. The number of terminal end buds (TEBs) was counted in the mammary glands at PD 22. Whole mounts of females PD 50 were only evaluated for density due to the large gland size and overlapping branches hampering outgrowth measurements and TEB number assessment.

2.6. Onset of puberty

Onset of puberty was registered in all weaned male and female offspring. In female offspring sexual maturity was assessed by determining day of vaginal opening (VO) as described by Goldman et al. [30]. All weaned females were examined daily from PD 30 to PD 42. In male offspring the onset of puberty was assessed as time of preputial separation (PPS) [31,32]. Males were examined daily from PD 34 to PD 50. On the day of VO or PPS the age and weight of the animals were recorded.

2.7. Behavioral testing

The investigations were performed during the animals' dark cycle, i.e. their active period, from 9 a.m. to 4 p.m., in dimly lit rooms. The experimenter was kept unaware as to which group an individual rat belonged, and exposed and control

Table 1
Composition of pesticide mixture.

Group	n[a]	Epoxiconazole	Mancozeb	Prochloraz	Tebuconazole	Procymidone	Pesticide mixture
1: Control	22(15)	–	–	–	–	–	–
2: Pestimix-14.6	22(17)	1.25	2.08	2.92	4.17	4.17	14.6
3: Pestimix-29.2	22(9)	2.5	4.17	5.83	8.33	8.33	29.2
4: Pestimix-43.8	22(14)	3.75	6.25	8.75	12.5	12.5	43.8
5: Epoxi-3.75	12(8)	3.75	–	–	–	–	
6: Epoxi-15	10(4)	15	–	–	–	–	
7: Manz-6.25	12(5)	–	6.25	–	–	–	
8: Manz-25	10(7)	–	25	–	–	–	
9: Prchl-8.75	12(8)	–	–	8.75	–	–	
10: Prchl-35	10(4)	–	–	35	–	–	
11: Tebu-12.5	12(8)	–	–	–	12.5	–	
12: Tebu-50	10(6)	–	–	–	50	–	
13: Procy-12.5	12(7)	–	–	–	–	12.5	
14: Procy-50	10(4)	–	–	–	–	50	

The number of dosed dams (viable litters) in each group and the doses of the pesticides, that were administered individually and in mixture in mg/kg/day, are shown in the table. Epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu) or procymidone (Procy).

[a] The large difference between the number of dosed dams and viable litters was mainly due to very low pregnancy rate from the animal breeders, and not to high rates of pre- and postnatal deaths in the offspring.

animals were tested alternately as were female and male animals. All statistics on behavioral data were conducted on litter means, and therefore the presented *n*-values describe the number of litters in each dose group.

2.7.1. Motor activity and habituation capability

In the tests of motor activity, all weaned offspring (140 males and 138 females) were tested ($n = 9–17$ for control and mixture groups and $n = 2–9$ for single pesticide groups). The offspring were tested on PD 28 and again at PD 59. The motor activity of the animals was recorded in activity boxes with photocells for 10×3 min (as described in Axelstad et al. [33]). The total activity during the 30 min was used as a measure of general activity. In order to assess habituation capability, the 30 min was divided into two time periods of 15 min.

2.7.2. Learning and memory (Morris Water Maze)

The number of animals used for the test of spatial learning, was somewhat smaller than for activity testing, as 90 males and 90 females were used ($n = 9–10$ for control and mixture groups and $n = 2–6$ for single pesticide groups). The animals were tested at age of 4–5 months in a maze with a diameter of 220 cm, filled with water at room temperature, as described in [33,34]. A circular transparent platform was situated on a solid support and submerged below the water surface. When the rat swam to and climbed onto the platform, the trial was completed. If the animal failed to locate the platform within 60 s, it was led to the platform. A video-tracking device (Viewpoint video tracking system, Sandown Scientific, Middlesex, England) was used to collect data about latencies to find the platform, the path lengths and swimming speeds of the animals.

Learning was tested with the platform situated at the center of the southwest quadrant of the pool, and the animals were trained in 4 trials per day for 7 days (five consecutive days, two day break and then two more days of training). Reversal learning was tested with a new platform position, the day after the last learning day. The animals were tested in a reversal procedure with the platform placed opposite the original location, again with 4 trials a day, but only for two consecutive days, i.e. days 8 and 9.

2.7.3. Mating behavior in male offspring

The males tested for mating behavior were the animals previously tested in the Morris Maze. For the assessment of male mating behavior, non-ovariectomized sexually mature female Wistar rats (weight 170 ± 20 g) were purchased from Taconic Europe. These female rats were treated with β-oestradiol-3-benzoate (25 μg/rat) 48 h before the mating and progesterone (500 μg/rat) 4 h before mating to obtain 'chemical estrus'. Both solutions were injected subcutaneously. The effects of progesterone dosing lasts for about 4 h and the females could therefore be used several times. Male rats need to be sexually experienced before a successful mating session can take place [35]. Therefore the males were placed with a female in 'chemical estrus' for 20 min before the test trial. It was required that the female showed proceptive behavior (described below) otherwise it was replaced by another female rat. The male rats had one training trial before the mating behavior test session, which was scored as described below.

The mating test was performed between 12.00 and 16.00 (in the active period of the animals) and the female rats were brought into 'chemical estrus' in relation to that time point. The male from the couple was placed in a transparent polycarbonate cage ($59.5 \times 38 \times 20$ cm ($D \times W \times H$)) with a flat lid and with no bedding in an unlit room. A female in "chemical estrus" was then introduced into the male cage and allowed to stay for 20 min. The same cage was used for all animals in the study. The mating behavior was recorded from the side of the cage using a standard Phillips

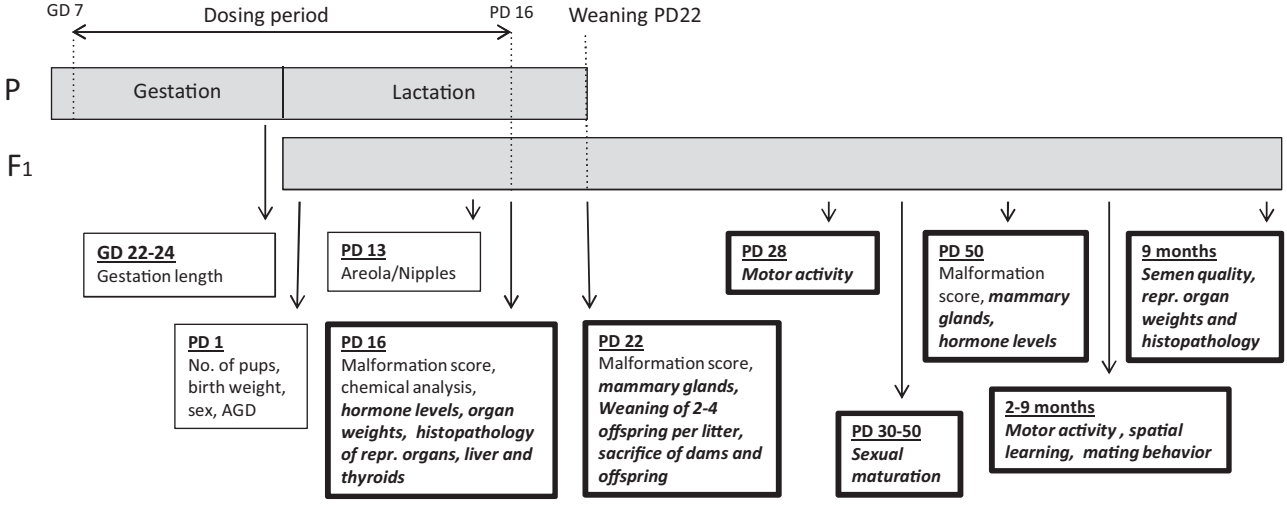

Fig. 1. Schematic overview of the design of the mixture study. Pregnant dams were dosed from gestation day 7 to postnatal day 16 with a mixture of pesticides, or the individual chemicals. In the present paper the results from organ weights, histopathology, mammary glands, hormone levels, timing of sexual maturation, behavioral tests and semen quality are presented (shown in italic in highlighted black boxes). The results from endpoints before PD 16 as well as malformation scores are presented in Hass et al. [28].

CCD-MOS video camera (black and white) with sensitivity in the infra-red area (800–950 nm). The recordings were stored on a hard disk/DVD recorder (LVW-545 HDD+DVD recorder).

All mating behavior data were scored using a Psion Workabout (ProInfo) with the software Pocket Observer® (Noldus, The Netherlands) installed. This was done by trained observers blind to experimental groups. The observer looked at the recordings of mating behavior on a computer and registered the behavioral elements Mount, Intromission and Ejaculation.

2.8. Necropsy of adult female offspring

Female offspring were autopsied at approximately 6 months of age. Stage of estrous cycle was determined using a rat Vaginal Impedance Checker (Model MK-10C (Muromachi, Japan). When an impedance value was higher than 3, the female was assumed to be in proestrus and was sacrificed. Animals were decapitated in CO_2/O_2 anesthesia and trunk blood was collected for hormone analysis. Thyroids (on the thyroid cartilage) were fixed in formalin and later embedded in paraffin. Histopathological evaluation was made of the thyroid. One section of the thyroid from rats belonging to groups 1, 4, 7 and 8 was stained with hematoxylin and eosin for histological evaluation by an examiner blinded to treatment groups.

2.9. Necropsy of adult male offspring

Sexually mature male offspring (261–280 days of age to around 9 months of age) were weighed, anesthetized in CO_2/O_2 and decapitated, and trunk blood was collected for hormone analysis.

Semen motility was analyzed in all dose groups, whereas sperm counts were only performed in the control males and in the males from the highest mixture dose group. For sperm motility analysis, spermatozoa were obtained from the distal cauda epididymis (alternately left and right) and sperm samples were prepared and analyzed by computer assisted sperm analysis (CASA) as described in Jarfelt et al. [36]. For sperm count analysis, cauda epididymis was weighed and prepared as described by Jarfelt et al. [36], and samples were analyzed using 10× UV fluorescent objective and IDENT OPTIONS. Ten fields were analyzed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda.

The rats were further autopsied and macroscopically examined. The following organs were excised and weighed: right and left testis, ventral prostrate, seminal vesicles with seminal fluid, epididymis, bulbourethral glands, LABC, liver and thyroid.

The right or left testis (alternately) was fixed in Bouin's fixative, paraffin embedded and stained with hematoxylin and eosin. The epididymis not used for motility analysis was excised and weighed.

The following organs were fixed in formalin and subsequently embedded in paraffin: caput epididymis, ventral prostrate, seminal vesicles and liver. One section per organ was stained with hematoxylin and eosin for histological evaluation by an examiner blinded to treatment groups. In ventral prostate the degree of epithelial atrophy, epithelial infolding and inflammation was scored in the following way: Epithelial atrophy: Score 0: no or minimal epithelial atrophy (≤5 acini affected); Score 1: moderate epithelial atrophy (>5% and <50% of the section affected); Score 2: marked epithelial atrophy (≥50% of the section affected). Papillary growth: Score 1: mild; Score 2: moderate; Score 3: marked. Inflammation: Score 1: no or very few scattered interstitial mononuclear cells; Score 2: focal to few multifocal interstitial accumulations of few mononuclear cells; Score 3: multifocal interstitial accumulations of mononuclear cells; Score 4: diffuse interstitial infiltrations of mononuclear cells. Additionally, prostates were evaluated for intraepithelial vacuolation, acini with concretions in the lumen, presence of areas with multilayering of epithelial cells, and focal acinar atrophy.

Ventral prostates from animals in dose groups 1 and 4 were further evaluated with regard to acini with atypical hyperplasia: score 0: no acini; score 1: few acini (<5% acini with atypical hyperplasia); score 2: moderate level (>5% and <50% of acini with atypical hyperplasia); score 3: marked level (>50% acini with atypical hyperplasia), acini with cribriform pattern: score 0: no acini; score 1: single acinus affected; score 2: 2–4 acini affected; score 3: >4 acini affected, and acini with columnar epithelium and papillary growth: score 0: no acini affected; score 1: <25% acini affected; score 2: >25% and <50% acini affected; score 3: >50% and <75% acini affected; score 4: >75% acini affected.

2.10. Hormone analysis

Trunk blood for hormone analysis was collected in Na–heparine coated tubes and centrifugated for 10 min, 4000 rpm at 4 °C. Plasma samples were stored at −20 °C. Progesterone, testosterone, and estradiol levels were analyzed in rat plasma at PD 16, PD 50/51 and in the dams at PD 22. T4 was measured in male and female pups at PD 50. The progesterone levels were analyzed in plasma from 1–5 male and 1–3 female pups in 4–5 litters per dose group. Testosterone and estradiol were analyzed in plasma from 1–3 male or 1–3 female pups in 3–5 litters, respectively. Plasma samples from the pups in each litter were pooled by sex. Furthermore, testosterone and progesterone levels were analyzed in plasma from 9 months old male and female offspring, respectively. Testosterone, estradiol, and progesterone were extracted from the plasma on IST Isolute C18 SPE columns as previously described [12] and samples were resuspended in heptanes. All hormones including T4 were analyzed using Delfia time-resolved fluorescence kits (PerkinElmer Life Sciences, Turku, Finland), and measured by use of a Wallac Victor 1420 multilable counter (PerkinElmer Life Sciences, Turku, Finland).

2.11. Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Data with normal distribution and homogeneity of variance were analyzed using analysis of covariance (ANCOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means.

Dunnett's test corrects for multiple comparisons, but applying this test on a study with 14 groups may lead to over-compensation and may lead to false negative results. For the analysis of reproductive organ weights an alternative approach to using a Dunnett's post hoc test on each group of chemicals separately was applied (i.e. separate analysis of controls and mixture groups 2–4, and separate analysis of controls and the two doses of each of the single pesticides). This separation of the study into one mixture study and five studies on single pesticides increases the likelihood of finding statistically significant differences, but also increases the risk of false positive findings. Results of both approaches are evaluated bearing these differences in mind.

A similar approach was taken in analysis of the behavioral data, however here the statistical analysis of controls and the two doses of the individual compounds were not performed, because of the low number of animals tested in these groups. The statistics on the Morris Maze data (i.e. swim length, swim speed and latencies) were calculated both for each separate test day, and for the combined total swim length and total latency, as this equals a 'repeated measures' test of these endpoints

Data obtained from prostate- and thyroid histology were analyzed statistically by Fisher's exact test. Prostate data from each mixture groups as well as each single compound groups were compared with the control group. If statistically significant differences ($p < 0.05$) were observed when all scores for a given endpoint were included, then individual scores were compared separately. For the densities of the mammary glands a one-way ANOVA with heterogeneous variance was used. For mammary gland densities PD 50, a threshold based on densities of control animals was set and a Fisher's exact test was used to make group comparisons of the numbers of animals with densities above this threshold.

Asterisks in tables and figures, indicate a statistically significant difference compared to controls *: $p \leq 0.05$; **: $p < 0.01$. ***: $p < 0.001$. All analyses were performed using SAS Enterprise Guide 3.0, SAS Institute Inc, Cary, NC, USA.

3. Results

3.1. Pregnancy, litter and offspring data

None of the applied exposures caused adverse effects on dam body weight gain, litter size or pup mortality. For further information regarding pregnancy and litter data please consult Hass et al. [28].

3.2. Section PD 16, organ weights

Absolute weights of male reproductive organs are listed in Table 2. In the comparisons of absolute organ weights, body weight was taken into consideration as this was included as a covariate in the statistical analysis. Reduced absolute weights of epididymis, prostate and seminal vesicle were seen in the highest mixture dose group compared with controls, in a model using Dunnett's post hoc test on all 14 dose groups. Epididymis weight was dose-dependently reduced in all three mixture groups compared with controls, though this effect was only statistically significant in the low and high mixture groups due to fewer animals in the middle dose group. Additionally, a statistically significant increase in absolute prostate weight was seen in animals exposed to the highest dose of epoxiconazole. Possible differences in weights of LABC, glandula bulbourethralis, liver and thyroid were not statistically significant in a Dunnett's test on all 14 groups. Comparable effects

Table 2
Absolute male organ weights on PD 16 and in adult male rats (PD 260–280) exposed to the pesticides singly or in mixture during fetal and neonatal life.

Absolute organ weights (mg)	*n*	Body weight (g)	Testis (g)	Epididymis (g)	Prostate (g)	Seminal vesicle (g)	LABC (g)	Bulbo (g)	Liver (g)	Thyroid (mg)
Male offspring PD 16										
1: Control	15	30.8 ± 5.7	103 ± 15	23.3 ± 2.3	10.6 ± 2.5	10.4 ± 3.6	26.5 ± 5.8	1.7 ± 0.4	786 ± 128	4.4 ± 0.7
2: Pestimix-14.6	16	28.9 ± 2.8	101 ± 11	**20.3 ± 2.5**$^{*,\#\#}$	8.8 ± 1.7	8.0 ± 2.0	22.7 ± 3.8	1.5 ± 0.4	735 ± 68	4.0 ± 0.6
3: Pestimix-29.2	9	30.2 ± 3.9	**109 ± 13**$^{\#}$	**20.8 ± 2.2**$^{\#}$	**8.5 ± 1.9**$^{\#}$	8.6 ± 1.8	26.4 ± 6.7	1.5 ± 0.4	811 ± 120	4.4 ± 0.8
4: Pestimix-43.8	12	30.7 ± 4.5	**110 ± 15**$^{\#}$	**19.4 ± 1.7**$^{***,\#\#\#}$	**7.1 ± 1.9**$^{***,\#\#\#}$	**7.2 ± 1.8**$^{**,\#\#}$	24.3 ± 5.6	1.5 ± 0.6	811 ± 123	4.4 ± 1.6
5: Epoxi-3.75	6	30.9 ± 4.5	106 ± 7	22.3 ± 1.6	10.9 ± 1.7	9.5 ± 1.0	26.1 ± 15.5	2.2 ± 0.8	789 ± 135	4.8 ± 1.4
6: Epoxi-15	3	33.7 ± 4.0	120 ± 10	**27.6 ± 0.9**$^{\#\#}$	**14.9 ± 2.7**$^{*,\#}$	9.8 ± 3.0	25.1 ± 3.5	2.2 ± 0.6	902 ± 87	4.3 ± 1.0
7: Manz-6.25	5	28.5 ± 3.6	100 ± 8	21.0 ± 2.0	9.6 ± 1.0	10.6 ± 2.2	23.4 ± 2.8	1.7 ± 0.4	718 ± 99	4.4 ± 0.7
8: Manz-25	7	27.2 ± 1.1	97 ± 7	22.6 ± 1.5	9.8 ± 3.1	8.6 ± 2.2	25.7 ± 4.7	1.7 ± 0.3	690 ± 38	4.3 ± 0.9
9: Prchl-8.75	9	30.2 ± 2.0	108 ± 8	23.3 ± 2.5	11.4 ± 0.8	8.6 ± 2.0	25.7 ± 3.2	1.6 ± 0.4	765 ± 77	3.8 ± 0.6
10: Prchl-35	4	30.0 ± 3.5	107 ± 19	**20.1 ± 2.6**$^{\#}$	10.8 ± 2.4	7.8 ± 1.8	22.8 ± 2.5	1.5 ± 0.5	760 ± 130	**3.4 ± 0.4**$^{\#}$
11: Tebu-12.5	8	28.7 ± 3.8	104 ± 16	22.4 ± 2.4	12.0 ± 2.6	9.8 ± 3.2	25.0 ± 3.6	1.7 ± 0.4	746 ± 96	4.1 ± 1.2
12: Tebu-50	5	30.5 ± 2.8	105 ± 8	22.0 ± 2.7	9.8 ± 2.9	11.5 ± 2.2	24.5 ± 2.2	1.7 ± 0.3	**839 ± 99**$^{\#\#}$	5.3 ± 1.2
13: Procy-12.5	6	29.8 ± 1.0	**112 ± 7**$^{*,\#\#}$	22.3 ± 3.5	8.6 ± 1.5	7.6 ± 1.4	27.8 ± 6.3	1.5 ± 0.2	786 ± 46	4.3 ± 0.9
14: Procy-50	3	28.1 ± 4.6	102 ± 16	**18.7 ± 3.0**$^{\#}$	**6.5 ± 0.7**$^{\#}$	8.2 ± 1.0	18.5 ± 2.4	**1.1 ± 0.4**$^{\#}$	771 ± 194	4.2 ± 0.6
Adult male offspring										
1: Control	16	497 ± 34	3.89 ± 0.31	0.70 ± 0.07	0.63 ± 0.15	1.91 ± 0.37	1.38 ± 0.19	0.22 ± 0.07	13.2 ± 1.4	22 ± 3
2: Pestimix-14.6	18	**458 ± 29**$^{\#}$	3.88 ± 0.24	0.69 ± 0.07	0.67 ± 0.17	1.94 ± 0.30	1.27 ± 0.17	0.24 ± 0.08	11.6 ± 0.9	24 ± 10
3: Pestimix-29.2	12	469 ± 50	4.03 ± 0.69	0.74 ± 0.11	0.63 ± 0.12	2.11 ± 0.40	1.28 ± 0.19	0.18 ± 0.07	12.0 ± 1.8	24 ± 4
4: Pestimix-43.8	16	457 ± 32	4.01 ± 0.26	0.69 ± 0.06	**0.47 ± 0.19**$^{\#}$	2.08 ± 0.38	**1.15 ± 0.23**$^{*,\#}$	0.21 ± 0.06	12.0 ± 1.2	20 ± 3
5: Epoxi-3.75	10	472 ± 41	4.00 ± 0.17	0.66 ± 0.07	0.63 ± 0.14	1.94 ± 0.35	1.24 ± 0.17	0.21 ± 0.05	11.9 ± 0.6	20 ± 2
6: Epoxi-15	2[a]	396 ± 31	4.06 ± 0.03	0.75 ± 0.001	0.64 ± 0.13	2.09 ± 0.001	1.28 ± 0.08	0.29 ± 0.06	11.4 ± 0.1	23 ± 1
7: Manz-6.25	8	464 ± 55	3.79 ± 0.27	0.69 ± 0.03	0.53 ± 0.25	1.82 ± 0.46	1.30 ± 0.28	0.22 ± 0.07	12.0 ± 1.6	30 ± 22
8: Manz-25	10	478 ± 36	3.89 ± 0.34	0.72 ± 0.08	0.61 ± 0.14	1.90 ± 0.43	1.23 ± 0.09	0.21 ± 0.07	12.9 ± 1.1	29 ± 15
9: Prchl-8.75	10	492 ± 30	4.15 ± 0.25	0.71 ± 0.05	0.64 ± 0.15	2.05 ± 0.25	1.31 ± 0.21	0.28 ± 0.06	12.9 ± 1.6	22 ± 3
10: Prchl-35	6	**437 ± 18**$^{\#}$	3.69 ± 0.19	0.64 ± 0.03	0.65 ± 0.11	1.98 ± 0.20	1.31 ± 0.20	0.22 ± 0.09	10.7 ± 0.9	21 ± 2
11: Tebu-12.5	8	**455 ± 27**$^{\#}$	3.84 ± 0.41	0.69 ± 0.12	0.59 ± 0.13	1.80 ± 0.29	1.24 ± 0.16	0.17 ± 0.06	11.3 ± 0.8	21 ± 2
12: Tebu-50	8	481 ± 62	3.83 ± 0.22	0.66 ± 0.03	0.55 ± 0.11	1.92 ± 0.41	1.23 ± 0.19	0.16 ± 0.02	12.7 ± 1.9	24 ± 4
13: Procy-12.5	8	481 ± 62	4.02 ± 0.43	0.73 ± 0.09	0.52 ± 0.10	1.95 ± 0.25	1.21 ± 0.21	0.18 ± 0.06	12.3 ± 1.3	21 ± 3
14: Procy-50	6	481 ± 47	4.03 ± 0.35	0.72 ± 0.07	**0.43 ± 0.19**$^{\#}$	1.73 ± 0.25	1.16 ± 0.22	0.21 ± 0.07	**11.5 ± 2.0**$^{**,\#}$	20 ± 4

Bulbo: glandula bulbocavernosus; LABC: levator ani/bulbocavernosus muscle.
Statistically significant differences between controls and exposed are marked with asterisks which indicate significance levels: *indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$. p values result from ANCOVA using body weights as a covariate followed by Dunnett's test on all 14 groups. # Indicates significantly different from controls in a model including only control and different doses of the same compound or the mixture using Dunnett's post hoc test. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. All significant results are written in bold. Epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu) or procymidone (Procy). [a] Data for adults in the Epoxi-15 group were left out of statistical analysis due to only 2 animals in this group.

were seen for analyses of relative organ weights (Table S1, Supplementary data).

In addition to the Dunnett's test on 14 groups, an alternative approach using a Dunnett's post hoc test on each group of chemicals separately was also applied, as described in the statistics section. Using this approach, epididymis weights were reduced in all three mixture groups, prostate weights were reduced and paired testis weights increased in the two highest mixture groups and seminal vesicles were reduced in the highest mixture group, compared to controls. Furthermore, statistically significant changes in organ weights were also seen for high doses of some of the individual pesticides compared with controls. The high dose of epoxiconazole increased epididymis and prostate weights, the high dose of prochloraz decreased epididymis and thyroid weights, the high dose of tebuconazole increased liver weights, the high dose of procymidone reduced weights of epididymis, prostate and the bulbo urethral gland, while the low dose procymidone increased paired testes weights (Table 2).

Because some of the single pesticide groups were quite small, an additional statistical approach was used, where data from the low and the high dose groups for each chemical were pooled, increasing the number of litters per chemical group. No statistically significant changes could be observed between controls and individual chemical groups, except for an increase in testis weight in the pooled procymidone group (data not shown). It may be noted that in some of these pooled chemical groups the n was still somewhat lower than the n of 12 litters in the high dose mixture group ($n = 9–13$, for exact number please consult Table 2).

In female offspring no changes in absolute or relative weights of uterus, ovary or liver were seen. Furthermore no statistically significant changes in body weights compared to control were observed (data not shown).

3.3. Section on PD 16, histology

No clear alterations were observed in the histological examination of testes, seminal vesicles and epididymides. Thyroid histology of controls and all three mixture groups was evaluated in female offspring, and no clear differences between groups were observed with respect to markers of activity, i.e. epithelial height and amount of colloid (data not shown).

3.4. Section adult offspring, organ weights

Absolute weights of male reproductive organs are listed in Table 2 and relative weights can be found in Table S1, Supplementary data. In the comparisons of absolute organ weights, body weight was taken into consideration as this was included as a covariate in the statistical analysis. Data from the high dose epoxiconazole group were omitted from the statistical analysis because only two males were available for data analysis. Reduced absolute weights of LABC were seen in the highest mixture dose group, using both statistical approaches. When the groups were analyzed separately, significant reductions were also seen on prostate weight in the highest mixture group and in the highest procymidone group. Body weight appeared slightly reduced in most dose groups though this was not statistically significant in the statistical approach using Dunnett's test on all 14 groups. When separating the dataset into single compound groups the reduction in body weight was statistically significant in the low mixture group, high prochloraz, and low tebuconazole groups.

The low body weight observed in mainly the epoxiconazole groups resulted in increased relative, but not absolute, weights of testes in the low epoxiconazole group (Table S1, Supplementary data). Statistical analysis of relative organ weights also revealed significantly decreased relative liver weight in the groups exposed to the high doses of prochloraz and procymidone, whereas absolute liver weights analyzed with body weight as a covariate were not significantly decreased by the high dose of prochloraz but only by the high dose of procymidone.

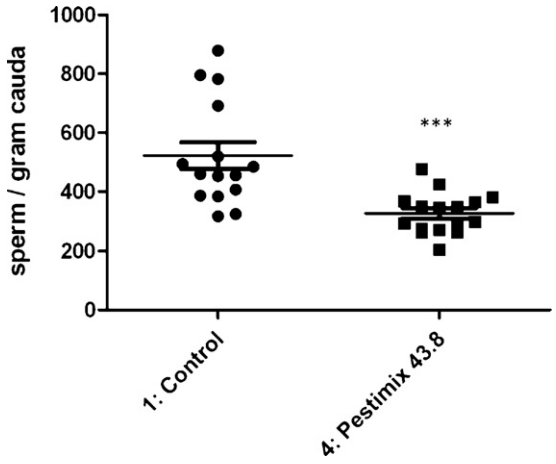

Fig. 2. Number of sperm per gram cauda epididymis in the adult males from the control group, and the group dosed with the highest dose of the pesticide mix during gestation and lactation. The control group included measurements from 15 males (representing 13 litters) and the mixture group included data from 16 males (representing 14 litters). Group means ± standard deviations for the two dose groups were 522.0 ± 178 and 326.4 ± 69.2, respectively. $^{***}p < 0.001$.

Thyroid weights were not affected by treatment. One animal from each of the mancozeb groups and one animal from the low dose mixture group had more than double thyroid weight compared to controls and to other pesticide exposed animals, but no treatment-related effects were observed with or without these outliers.

No dose related effects were observed on weights of uterus, ovary or liver in adult female offspring (data not shown).

3.5. Semen quality

The sperm count in the adult males was significantly lowered in the highest mixture group compared to controls ($p < 0.0005$) (Fig. 2). The sperm count results are shown as number of sperm per gram cauda epididymis, and the observed reduction was not due to effects on the weight of the cauda epididymis, as this was unaffected by the exposure (data not shown). Sperm motility parameters were investigated in all 14 dose groups, but neither % motile sperm nor % progressive sperm were significantly affected by pesticide exposure (data not shown).

3.6. Section adult offspring, histology

Testicular histology appeared normal in most animals, whereas two animals had testes with tubular degeneration in approximately 20% of seminiferous tubule cross-sections. As this finding may also be seen in controls and as these animals were from two different low dose groups (tebuconazole and procymidone), this was not considered to be dose related (data not shown). Due to the finding of reduced sperm number in the high mixture group, the histological examination was repeated on a new set of testicular sections (high mixture group and controls only) including the rete testis to see if examination of the rete testis would reveal subtle changes in histology. This examination showed no differences between dose groups (data not shown).

Caput epididymides were examined in groups 1 and 4, and both groups had normally appearing epithelium, equally high sperm

content, and had no sloughed cells or cell debris in lumen (data not shown).

In the ventral prostate, various lesions were observed in both controls and exposed animals, and acinar epithelial atrophy was observed less frequently in the high dose mixture compared with the control group (Table 3). In the single compound groups, no statistically significant differences were found with respect to scores for epithelial atrophy (data not shown). Increasing scores for papillary growth were found in a few dose groups (low dose mixture and low dose prochloraz) compared to controls (data not shown). No statistically significant differences were seen with respect to the following findings: interstitial inflammation, intraepithelial vacuolation, acini with concretions in the lumen, presence of areas with multilayering of epithelial cells, and focal acinar atrophy. The observation of lower scores for atrophy in the high dose mixture was followed up by an evaluation of hyperplastic lesions including presence of atypical hyperplasia and cribriform patterns in controls and the high dose mixture group. Animals in the high dose mixture group showed higher scores for atypical hyperplasia and cribriform pattern ($p = 0.011$ and $p = 0.031$, respectively, Table 3) compared with controls.

In the seminal vesicle a large degree of variation in lumen size, epithelial height, epithelial infolding and secretory content were seen, and overall, no treatment-related findings were observed (data not shown).

The activity of the thyroid was generally not very marked in the adult female offspring. Follicles with high columnar vacuolated epithelial cells indicating higher degree of secretory activity were only observed in few animals, mainly belonging to the high dose mancozeb group. Correspondingly, a statistically significant increase in the number of animals with follicles dominated by columnar to cuboidal epithelium was seen in mancozeb exposed animals compared to the control group (observed in 4 of 9 animals from the high dose mancozeb group compared to 1 of 17 control animals, $p = 0.03$). Mild diffuse C-cell hyperplasia was observed in thyroids of all groups with no statistically significant differences.

No clear differences were observed in liver histopathology between controls and the exposed groups (data not shown).

3.7. Mammary glands PD 22 and 50

Examination of mammary gland whole mounts of males and females at PD 22 showed no statistically significant changes in longitudinal growth, transverse growth, glandular area, glandular density or in the number of terminal end buds (Table S2, Supplementary data). Histopathology of female mammary glands PD 50 showed a lobuloalveolar pattern in one animal from each of the 3 mixture groups (not statistically significant) (Table S2). At PD 50, mammary glands of both male and female rats showed higher densities with increasing mixture doses (Fig. 3 and Table S2). When evaluated using an ANOVA approach this apparent increase in density was not statistically significant. However, when considering the distribution of density scores, it was evident that density score 4 and 5 were only present in males of the mixture groups (Fig. 3). This increase in the incidence of high density scores for males was statistically significant in a Fisher's exact test comparing animals with scores above 3 in exposed groups versus controls ($p = 0.03$ in both high mixture groups). For females, a slightly higher frequency of scores 4 and 5 was seen in exposed animals than in controls, but this was not statistically significant. Male mammary glands PD 50 were also evaluated for growth and area. The longitudinal growth was increased with dosing and was statistically significantly higher in males PD 50 in group 3 ($p = 0.04$) (Table S2). No changes were found in the transverse growth and area of male mammary glands PD 50 (data not shown).

Table 3
Histological findings in adult prostates of rats exposed to the pesticide mixture during fetal and neonatal life.

	Epithelial atrophy score 0	Epithelial atrophy score 1	Epithelial atrophy score 2	Atypical hyperplasia score 0	Atypical hyperplasia score 1	Atypical hyperplasia score 2	Atypical hyperplasia score 3	Cribriform pattern Score 0	Cribriform pattern Score 1	Cribriform pattern Score 2	Cribriform pattern Score 3
1: Control	31% (5/16)	25% (4/16)	44% (7/16)	6% (1/16)	75% (12/16)	13% (2/16)	6% (1/16)	75% (12/16)	13% (2/16)	6% (1/16)	6% (1/16)
2: Pestimix-14.6	28% (5/18)	56% (10/18)	17% (3/18)	ND	ND	ND	ND	ND	ND	ND	ND
3: Pestimix-29.2	25% (3/12)	58% (7/12)	17% (2/12)	ND	ND	ND	ND	ND	ND	ND	ND
4: Pestimix-43.8	**81% (13/16)****	13% (2/16)	**6% (1/16)***	0% (0/16)	**31% (5/16)***	**63% (10/16)****	6% (1/16)	**25% (4/16)***	25% (4/16)	38% (6/16)	13% (2/16)

Values are percentage of affected animals, and in parentheses are given numbers affected of animals/total number of animals examined. Statistically significant differences between controls and exposed are marked with asterisks. All significant results are written in bold. Epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu), procymidone (Procy). ND: not determined.

* $p < 0.05$.

** $p < 0.01$.

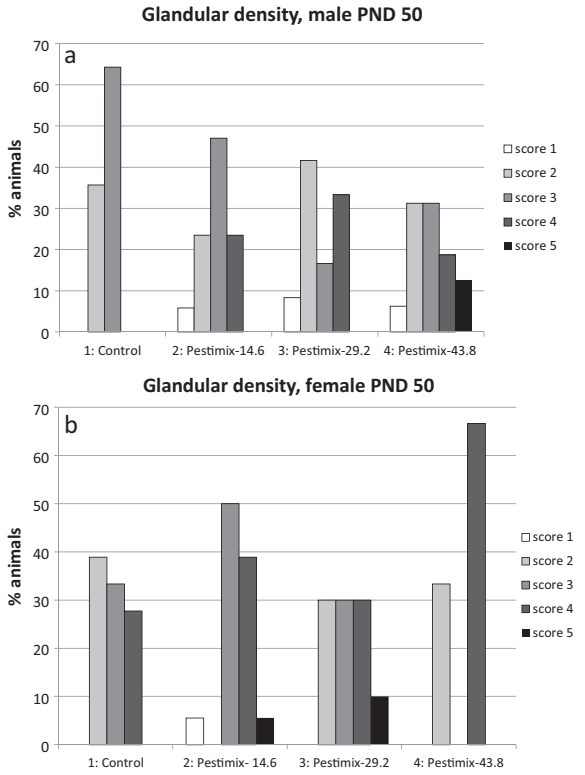

Fig. 3. (a,b) Mammary gland whole mounts PD 50. Glandular density was evaluated as described in Section 2 and the distribution of density scores is presented as % of animals with a given score for each group separately. For males, scores 4 and 5 are only present in exposed groups indicating a dose-related influence on mammary development in some individuals. Number of animals examined PD 50; males $n = 14$ (control), $n = 17$ (Pestimix-14.6), $n = 12$ (Pestimix-29.2), $n = 16$ (Pestimix-43.8), in female $n = 18$ (control), $n = 18$ (Pestimix-14.6), $n = 10$ (Pestimix-29.2), $n = 9$ (Pestimix-43.8).

3.8. Onset of puberty

No treatment related effects were observed in age or weight at onset of puberty determined as VO or PPS in either male or female offspring (data not shown).

3.9. Activity

In 28 day old offspring motor activity levels did not show any effects of pesticides exposure or any differences between male and female pups (data not shown). When the offspring were tested again in adulthood, there was a statistically significant sexual dimorphism, with females showing significantly higher activity levels than males ($p < 0.0001$) (Table S3, Supplementary data). The results from the two sexes were therefore analyzed separately. No statistically significant effects were seen in either males or females exposed to the pesticide mixture, compared with controls (Table S3, Supplementary data). In the animals dosed with the single pesticides, no significant effects were seen in the male offspring, whereas significantly higher activity level were observed in females from the high dose mancozeb group ($p = 0.007$), the low dose prochloraz group ($p = 0.029$) and low dose tebuconazole group ($p = 0.029$) (Table S3, Supplementary data). None of the observed effects were due to effects on habituation (data not shown).

3.10. Spatial learning

During the last four days of the learning period (days 4–7), a statistically significant sexual dimorphism in Morris Maze performance was observed, with females spending more time in the water and swimming further before finding the platform than males ($p < 0.0001$) (Table S4, Supplementary data). The results from the two sexes were therefore analyzed separately.

In Fig. 4, total swim lengths during the learning period (days 1–7) (Fig. 4a) and total latency to reach the platform during this period (Fig. 4b), are shown for male and female offspring from controls and the three mixture groups. In males, both endpoints showed a dose-dependent increase, which reached significance in the highest mixture dose group compared to controls, when all 14 groups were included in the statistical analyses ($p = 0.03$ and 0.02, for swim length and latency respectively). When the test was performed on just the data from the control and mixture groups, the p-values were no longer statistically significant but only borderline ($p = 0.11$ and 0.07 respectively). As shown in Fig. 5, the effect of pesticide exposure on male swim length and latency was primarily observed at the end of the learning period (days 6 and 7) and reached significance for swim latency on day 7, with high mixture group males spending significantly more time to find the platform than control males ($p = 0.01$). The picture was similar for swim length, but did not reach significance, even on day 7 (Table S4, Supplementary data). The effect on swim latency on day 7 was also significant when the data form control and mixture groups were analyzed separately ($p = 0.049$). The higher group mean values in the highest mixture group were primarily caused by a few animals still having very high latencies and swim length values at the end of the learning period, whereas all males in the control group had efficiently learned to find the platform at this time point. In the female offspring, there were no statistically significant effects of exposure to the mixture on either swim length or latencies to reach the platform (Fig. 4a and b), and in both sexes, no statistically significant effects of pesticide exposure were observed on the animals' swimming speed (data not shown).

In the female animals exposed to the single pesticides, no statistically significant effects were seen (Table S4, Supplementary data). In the males, statistically significant increases in total latency and swim length were seen only in the low dose tebuconazole group and the high dose procymidone group (see Table S4, Supplementray data). Like in the mixture groups, these increases were most marked on day 7 of testing.

No effects of pesticide exposure were seen in neither males nor females during reversal learning, on days 8 and 9 (data not shown).

3.11. Mating behavior

Mating behavior was assessed in 87 males (from all groups), as one of the 88 recordings was spoiled. No statistically significant differences in mating behavior (assessed as frequency of mounting, intromission and ejaculation, and as intromission latency) were revealed between the control and the three mixture groups. Moreover, no statistically significant differences between the control and the single pesticides groups in mating behavior were revealed (data not shown).

When the animals were characterized, as belonging to either a low (i.e. number of mounts below or equal to 5), medium (number of mounts between 5 and 30) or high (number of mounts equal to or above 30) group with regards to general mating activity, the males from the highest dose of prochloraz showed significantly increased mating activity ($p < 0.001$) compared to the controls. In Fig. 6, the distribution in these different groups in relation to mounting frequency is shown.

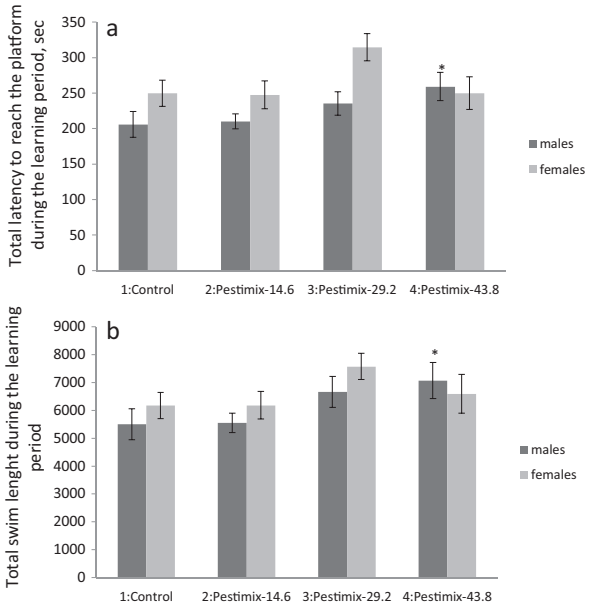

Fig. 4. (a,b) Total latency to reach the platform (a) and total swim length (b) in adult male and female offspring exposed to 0, 14.6, 29.2 or 43.8 mg/kg/day of the pesticide mixture during fetal and neonatal life. Data is shown as group means ± SEM for total latency and swim length to reach the platform in the Morris Water Maze, $n = 9–10$. Statistically significant differences between controls and exposed are marked with asterisks which indicate significance levels: $^{*}p < 0.05$. The results from these groups and the 10 single pesticide exposed are shown in Table S4, Supplementary data.

3.12. Hormone levels

There were no statistically significant effects of exposure to the mixture on any of the measured plasma hormone levels in dams or in male or female pups (data not shown).

Also, there were no statistically significant effects of exposure to the single pesticides on progesterone and testosterone levels in adult offspring.

4. Discussion

This study generally aimed at exploring whether combined perinatal exposure to low doses of five endocrine disrupting pesticides could lead to adverse reproductive and neurobehavioral effects, including effects observed months after ended exposure. It was also the intention to investigate whether effects of the mixture were present at dose levels where single compounds were ineffective, i.e. at NOAELs for single compounds. However, due to very low pregnancy rates, some dose groups were unintentionally small, and as discussed in the following, the reader should bear this in mind when interpreting the data on single compound effects. Alterations in reproductive organ weights and altered histology were seen in both young and adult offspring, and decreased sperm count and impaired spatial learning was observed in adult male offspring. Furthermore, reproductive organ weights changes generally showed cumulative effects, confirming the mixture effects also described by Hass et al. [28].

4.1. Mixture effects on reproductive organ weights

As can be seen in Table 2, weights of male reproductive organs in both 16 day old and adult offspring were affected by a combination of the 5 pesticides at dose levels where the individual pesticides caused no or only small effects. Fig. 7 shows the relative

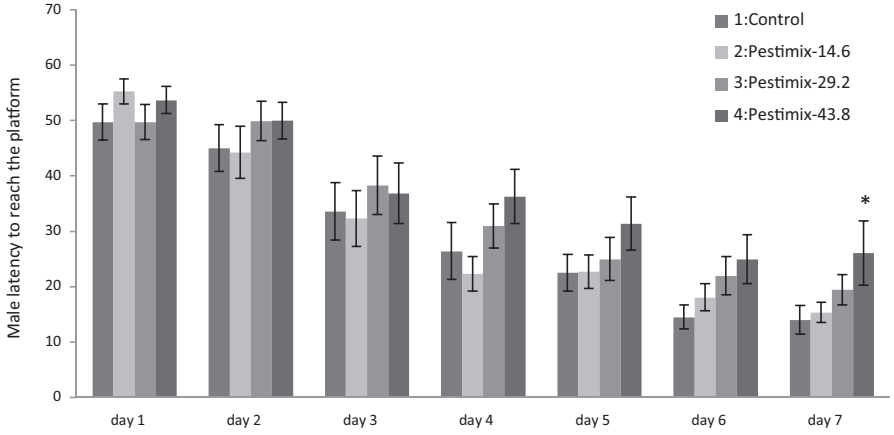

Fig. 5. Mean daily latency to reach the platform in adult male rat offspring exposed to 0, 14.6, 29.2 or 43.8 mg/kg/day of the pesticide mixture during fetal and neonatal life. Data is shown as group means ± SEM for latency in the Morris Water Maze for the first seven days of the learning testing, $n = 9–10$. Statistically significant differences between controls and exposed group on each day of testing are marked with asterisks which indicate significance levels: $^{*}p < 0.05$. The results from these groups and the 10 single pesticide exposed are shown in Table S4, Supplementary data.

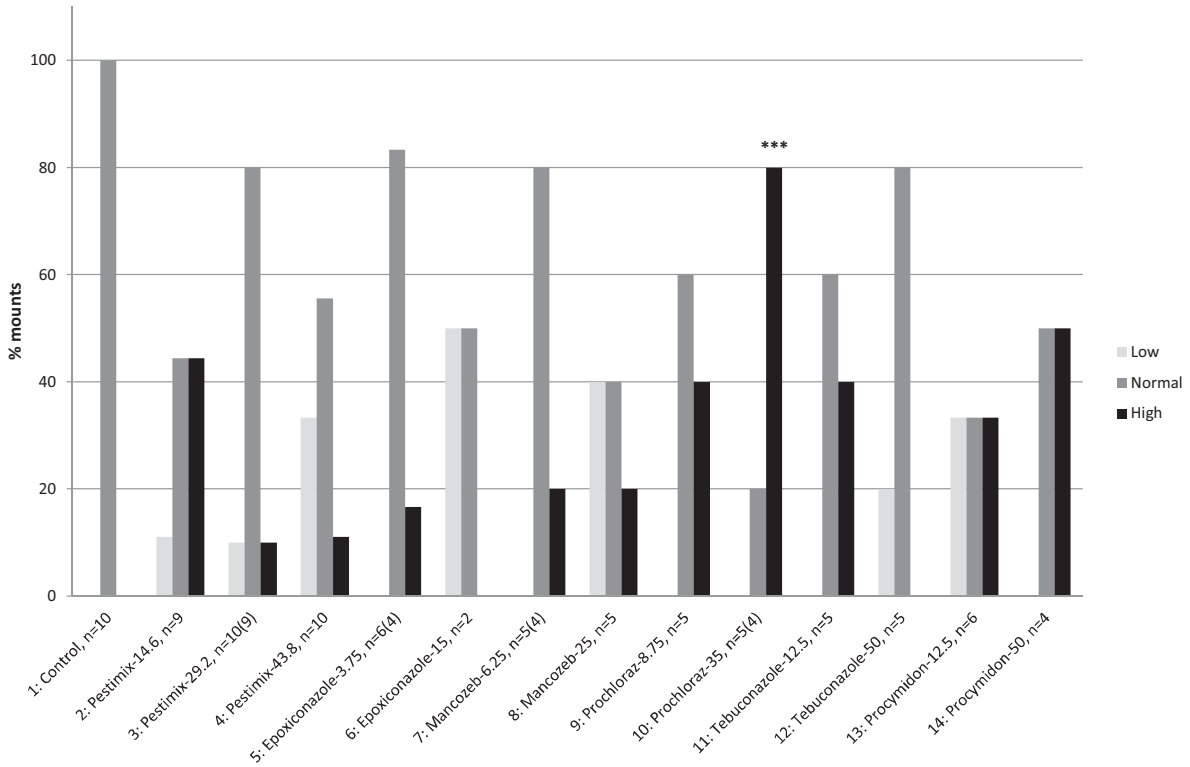

Fig. 6. Mating behavior, % mounting activity and distribution in 3 groups. Low (number of mounts ≤5), medium (number of mounts >5 and <30) or high (number of mounts ≥30), during a 20 min. period, shown as group means (%). The number of males is shown as *n*, while the number in parenthesis is the number of litters. ***$p < 0.001$.

epididymis, prostate and seminal vesicle weight data from day 16 offspring and relative prostate and LABC weights from adult males compared to control values. Results are shown for the highest mixture group and for the individual pesticides in the low doses, which were included in this mixture, and the figure illustrates how the slight effects of individual compounds add up to a marked effect in the mixture group at the same dose levels. Similar results have previously been obtained in another mixture study of three similarly acting anti-androgens, i.e. the androgen receptor (AR) antagonists flutamide, procymidone and vinclozolin [24]. For chemicals acting via the same mode of action, e.g. AR antagonists, effects will be in the same direction (e.g. decreased organ weights) and dose-additive effects are expected when such compounds are given in combination. Evidently, exposure to antagonists in combination with agonists for the same receptor, may result in opposite directions of effects and thus one compound compensating the effect of

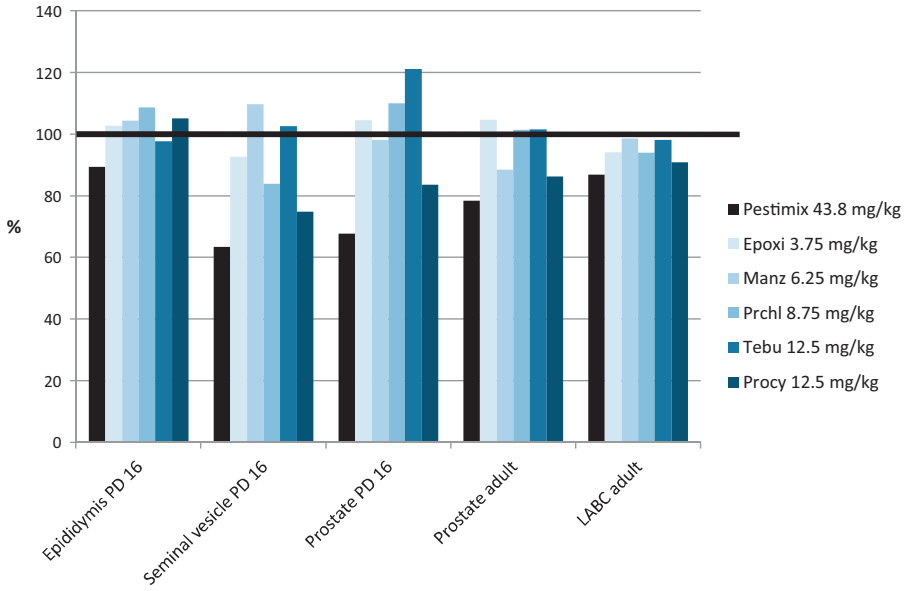

Fig. 7. Organ weight changes compared with controls in male rat pups exposed to the low dose of epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu), procymidone (Procy) or the highest mixture dose of these pesticides (Pestimix) from GD 7 to PND 16. Values are expressed as percent of control values for relative organ weights. Horizontal line indicates control level 100%. *n* = 2–16 as listed in Table 2.

another. For compounds with multiple modes of action, the effects of each chemical may be in opposite direction (increase versus decrease) and the effect of the mixture is more difficult to predict. In the current study, procymidone appears to be the dominating compound in the mixture as procymidone has the largest individual effect reducing prostate and seminal vesicle weights at the applied doses. In contrast, the low dose of tebuconazole slightly increased prostate weights and the high dose of epoxiconazole significantly increased prostate weights at PD 16. These azole fungicides have multiple modes of action, i.e. anti-androgenic, anti-estrogenic and steroid synthesis disrupting actions, which may influence prostate weight in opposing directions. However, when these azole fungicides were given in combination with procymidone, the joint effect is a significant reduction in prostate weight at a lower dose of procymidone than is effective alone. Currently, little is known about combination effects on endpoints influenced in opposite directions by endocrine disrupters with different modes of action and more research is needed in this area.

It should be noted that the low doses of the individual compounds were similar to the doses used in the highest mixture group. As the numbers of animals in the individual chemical groups were lower than the number of animals in the mixture groups, it could be argued that the low "*n*" of the individual chemical groups might reduce the likelihood for detecting statistically significant changes. However, even after pooling weight data from the low and the high dose groups for each chemical where "*n*" was increased to 9–13 litters per chemical group, no statistically significant changes could be observed between controls and individual chemical groups, except for an increase in testis weight in the pooled procymidone group. Thus, increasing the number of litters in the individual chemical low dose groups would not change the overall picture that effects on male reproductive organ weights occur in mixture groups but not in individual chemical groups using the same doses. The reductions in weights of male accessory reproductive organs on PD 16 in only the highest mixture group indicates a cumulative effect seen at dose levels where each compound does not have a statistically significant effect. Cumulative effects, in relation to mixtures toxicology, has previously been introduced by Silva et al. [23] who showed that in mixture studies, every chemical in the mixture, in proportion to its toxic unit, can contribute to the overall effect – even when it is present at concentrations below the threshold of statistically detectable effects. The concept has subsequently been proven several times during the last decade in both in vivo and in vitro studies [23,25,37–43].

A statistically significant increase in testis weights was observed in animals exposed to the lowest dose of 12.5 mg/kg bw/day of procymidone on PD 16 but not at 50 mg/kg bw/day. Interestingly, our own previous study on procymidone showed a statistically significant increase in testis weight at 10 mg/kg bw/day, no change at 25–100 mg/kg bw/day and a decrease at 150 mg/kg bw/day pointing to a non-monotonous dose–response curve [24]. Testis weight may be affected by chemically induced changes such as fluid accumulation or impaired proliferation/differentiation, and these changes will likely have opposing effects on testis weight. If these changes appear at different doses, it may be speculated that this could result in non-monotonous dose–response curves for testis weight.

In adult offspring, reduced absolute weight of LABC and prostate were seen in the highest mixture dose group compared to controls, but not in any of the groups exposed to the pesticides alone at the same dose levels, again confirming a cumulative effect seen at dose levels where each compound did not have a statistically significant effect (Table 2 and Fig. 7). The decreased weights of prostates in the high dose mixture group corresponded well with the finding of low weights of prostates, seminal vesicles and epididymides on PD 16 and revealed that pesticide effects on male reproductive organs persisted into adulthood. The decreased weight of the LABC in the adult but not prepubertal male offspring may indicate long-term delayed mixture effects, but could also reflect a higher degree of variation (biological or procedural) at PD 16 than in adulthood. No changes in seminal vesicle or epididymis weight were observed in adult animals, indicating that PD 16 may be a more sensitive time point for observing impaired growth of male reproductive organs due to anti-androgenic effects of these pesticides. Our own previous studies on perinatal exposure to other anti-androgenic chemicals have shown that prostate weight on PD 16 is often affected at low doses. For different anti-androgenic chemicals, it differs whether LABC, seminal vesicle or epididymis weights are also affected at the same doses that affect prostate weight, i.e. prostates appear to be most sensitive to anti-androgens whereas weights of other male reproductive organs vary in their sensitivity depending on the type of chemical exposure.

4.2. Effects on sperm count, semen quality and adult reproductive organs

Exposure to the pesticide mixture lowered sperm counts in the highest dose group. Control values did not differ from historical control values and our finding therefore indicates that the lowered sperm counts were caused by developmental exposure to this mixture of endocrine disrupting pesticides. This is a very important and potentially quite alarming result in relation to the low sperm counts and declining sperm quality in humans reported during the last decades [6,44]. No effects on sperm motility parameters were seen in the exposed animals in the present study, which indicates that the mixture affected the number but not the function of the sperm cells.

Histological evaluation of testes and caput epididymides showed no differences between controls and the high mixture group. The epididymis is well known to be a sensitive indicator of spermatogenic disturbance showing increased numbers of sloughed cells and cell debris in case of endocrine disruption such as anti-androgen effects [45]. However, in this study the effects were seen months after exposure, and any histological changes were likely to be much more subtle than would be expected from studies on direct exposure. It should also be noted that no histological examination of corpus or cauda epididymis was performed and that functional and/or histological changes in these parts may be present.

Although the weights of prostate in adult offspring were reduced in the highest mixture group compared to the control group, this was not associated with any increase in prostate atrophy. Interestingly, in the present study a higher degree of atypical hyperplasia and cribriform patterns together with less epithelial atrophy was observed in the highest mixture dose group compared with the control group. In rats, epithelial atrophy is a known age-related finding in prostate, probably caused by a decline in circulating testosterone [46]. This apparent change from epithelial atrophy (as a normal age-related finding) towards epithelial hyperplasia (as an abnormal change) may be related to endocrine disruption. It is generally agreed that early exposure to endocrine disrupting chemicals can initiate imbalances in the prostatic cells which may contribute to prostatic pathology with aging [47,48]. Neonatal exposure to bisphenol A resulted in an increased susceptibility to precancerous lesions in the adult rat ventral prostate [48]. An adult onset of mainly augmented atrophic changes and to a lesser extent hyperplastic changes were seen in aged ventral prostates through four generations of rats following neonatal exposure to Vinclozolin in F0 [47]. The findings of atypical hyperplasia and cribriform pattern in ventral prostates of the highest mixture dose group in the current study may similarly reflect alterations caused by early exposure to this pesticide mixture. A comparable shift from atrophy towards

hyperplasia of prostatic epithelium has been observed in another study on aging rats exposed perinatally to anti-androgenic chemicals ([47] manuscript in preparation).

4.3. Thyroid and liver effects

Since at least one (mancozeb) and possibly more of the tested pesticides (the azole pesticides) may affect the thyroid hormone system, several endpoints which can be used to investigate thyroid disruption were included in the present study.

On pup day 16, reduced thyroid weights were seen in animals exposed to the high dose of prochloraz. Similar effects have not been described in the literature and have not been investigated in our previous studies on prochloraz [11,13]. In adults an increase in the number of rats with follicles dominated by columnar to cuboidal epithelium was found in group 8 compared to the control group indicating thyroid hyperactivity. This confirms previous findings that Mancozeb produces structural and functional changes in rat thyroids [49,50].

No thyroid weight or histological changes were seen in mixture groups, and T4 levels were measured in male and female pups at PD 50 in control and mixture groups, and at this age no statistically significant changes were seen in any dose group, indicating that possible effects of prochloraz and mancozeb would require higher doses than those included in the mixture. Thyroid hormones are important to growth and mental development, and in the activity test, a significantly elevated activity level was seen in the high dose Mancozeb females. This could reflect a real effect on activity levels, however other studies on Mancozeb performed in our laboratory using a larger group size did not show similar effects [19], and this may therefore be considered a chance finding due to the relatively low number of offspring in the mancozeb group. Also, no changes in the learning and memory of the animals were observed in the Mancozeb groups. All in all, no significant changes indicating anti-thyroid properties of the pesticide mixture were observed.

The observed increase in absolute and relative liver weight observed at PD 16 in animals exposed to 50 mg/kg bw/day of tebuconazole were comparable to findings in a study by Moser et al. [51], who described increased liver weights at PD 46 in rats following perinatal exposure to 60 mg/kg bw/day of tebuconazol.

4.4. Effects on mammary glands

Mammary glands of offspring from the three mixture groups showed no altered growth or development on PD 22. On PD 50 increased densities of male mammary glands appeared in the high dose mixture groups. The female form of the mammary glands in rodents is the default morphology of the mammary glands and testosterone is responsible for the development of the male morphology of the mammary glands (Goldman et al. [52]). Hence, the anti-androgenic effects of the mixture in the present study may impart the virilisation of the fetal male mammary glands. The dose-related increase in density of the male mammary glands may thus reflect the anti-androgenic properties of the mixture. In addition, nipple retention is strongly linked to endocrine disrupting chemicals [25]. More nipples were found in the male offspring exposed to the mixtures, as reported in Hass et al. [28]. This finding may comply with the increased density in the male mammary glands PD 50 reflecting a possible permanent feminization of the mammary glands. Likewise, more male mammary glands in the high mixture group had secretory material in the ducts and more vacuolated epithelium, suggesting an increased secretory activity of the glands. This may be linked to an increased feminization of the male mammary glands. However, in some of the animals, it was not evident if the material in the ducts were of secretory origin. Further investigation with other staining techniques will be necessary to determine the nature of the material in the ducts.

4.5. Effects on onset of puberty

No effects were seen in age or weight at onset of puberty on male or female offspring in either the groups receiving the mixtures or the single chemicals in the present study. However, as some of the pesticides have been shown to affect timing of sexual maturation and several of the pesticides used in this study can act as anti-androgens [14,15,17,53,54] onset of puberty could have been affected in the tested animals [55].

4.6. Behavioral studies

In the behavioral studies of motor activity, no statistically significant exposure-related effects were seen in males or females from the three mixture groups, compared to controls. As behavioral studies conducted with the small group sizes which were present in the single pesticides groups are very difficult to interpret, the significantly elevated activity levels observed in female rats exposed to the low doses of prochloraz and tebuconazole and the high dose of mancozeb were probably random findings. No dose–response relationship was seen for the two first groups, and a previous study of mancozeb [19] did not show any effect on activity level using much larger group sizes and higher dose levels.

In the Morris Maze spatial learning test significant effects of mixed pesticide exposure were seen in the male offspring. On the last day of the learning period (day 7), males from the highest mixture group spent significantly more time in the maze and swam significantly longer than control males before reaching the platform, indicating decreased learning ability in these animals. Even though the effect was most marked on day 7, a similar dose-dependent increase in swim length and latency was seen over the last three days of the learning period. The fact that no effects on activity levels were seen in the males from three groups dosed with the pesticide mixture indicates that the significant effects seen in the Morris Water Maze were not related to alterations in the level of general motor activity. As male rats generally perform better than females in tests of spatial learning [56,57], a result that was also confirmed in the present study, the signs of decreased learning ability seen in the pesticide exposed males could be interpreted as being in the direction of female performance.

Spatial learning ability after perinatal pesticide exposure has previously been investigated for prochloraz, mancozeb and procymidone, but none of the compounds affected learning ability when tested alone, as a dose of 30 mg prochloraz/kg/day [11], doses of 50–100 mg mancozeb/kg/day [19] and doses of 25–100 mg procymidone/kg/day [27] did not result in any significant alterations of spatial learning ability in male or female offspring. However, demasulinization of another sexually dimorphic behavior, sweet preference, was seen in the males from the prochloraz study [11]. Moreover, spatial learning ability was impaired in males dosed perinatally with a mixture of the three anti-androgenic chemicals vinclozolin, flutamide and procymidone, as both tested doses of the mixture resulted in impaired Morris Maze performance in the adult male offspring [27]. The same mixture doses caused increased nipple retention as well as decreased weights of epididymis, ventral prostate and bulbouretral glands [24,25]. These results indicate that behavioral effects induced by combined exposure to anti-androgenic chemicals may be caused by altered sexual differentiation of the male brain. More studies are however needed before clear conclusions on this matter can be drawn.

For the single pesticide exposures, group sizes were generally quite small in regard to assessment of behavioral results

(n = 2–6). Therefore, conclusions on whether the statistically significant increase in swim length and latency seen in the low but not the high dose tebuconazole males, could be involved in the observed mixture effect in the males are only speculative.

In the behavioral studies of mating behavior, males from the highest dose of prochloraz showed significantly increased mating activity compared to the controls. However, due to the limited number of animals in the single pesticide exposure groups this finding can only be considered as suggestive.

4.7. Overall conclusions

The study showed that a mixture of the endocrine disrupting pesticides epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, was able to cause adverse developmental toxicity effects, including long-term delayed effects, at dose levels where the single pesticides had no effects. The adverse effects, which included decreased weight of reproductive organs, decreased sperm count, altered mammary gland development and decreased spatial learning ability in male offspring, were seen after exposure to a mixture, where the individual pesticides were present at dose levels below their respective NOAEL values. These results imply that risk assessment based on NOAELs for single chemicals can underestimate the risk, and that there is a need for modification of risk assessment procedures for pesticides in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction. This issue is discussed in much more detail in the paper by Hass et al. [28], and is supported by the present findings.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.05.099.

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ORIGINAL ARTICLE

Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats

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anti-androgens, bisphenol A, combined exposure, endocrine disrupters, mixtures, nipple retention, paraben, pesticides, phthalates, rat, UV-filters

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Summary

By diminishing the action of androgens during gestation, certain chemicals can induce irreversible demasculinization and malformations of sex organs in the male rat after gestational exposure. Studies with mixtures of such anti-androgens have shown that substantial combined effects occur even though each individual chemical is present at low, ineffective doses, but the effects of mixtures modelled based on human intakes have not previously been investigated. To address this issue for the first time, we selected 13 chemicals for a developmental mixture toxicity study in rats where data about in vivo endocrine disrupting effects and information about human exposures was available, including phthalates, pesticides, UV-filters, bisphenol A, parabens and the drug paracetamol. The mixture ratio was chosen to reflect high end human intakes. To make decisions about the dose levels for studies in the rat, we employed the point of departure index (PODI) approach, which sums up ratios between estimated exposure levels and no-observed-adverse-effect-level (NOAEL) values of individual substances. For high end human exposures to the 13 selected chemicals, we calculated a PODI of 0.016. As only a PODI exceeding 1 is expected to lead to effects in the rat, a total dose more than 62 times higher than human exposures should lead to responses. Considering the high uncertainty of this estimate, experience on lowest-observed-adverse-effect-level (LOAEL)/NOAEL ratios and statistical power of rat studies, we expected that combined doses 150 times higher than high end human intake estimates should give no, or only borderline effects, whereas doses 450 times higher should produce significant responses. Experiments indeed showed clear developmental toxicity of the 450-fold dose in terms of increased nipple retention (NR) and reduced ventral prostate weight. The 150-fold dose group exhibited significantly increased NR. These observations suggest that highly exposed population groups, especially women of reproductive age, may not be protected sufficiently against the combined effects of chemicals that affect the hormonal milieu required for normal male sexual differentiation.

Introduction

Normal development of the male reproductive tract depends on a balanced action of androgens and oestrogens (Hotchkiss *et al.*, 2004; Delbes *et al.*, 2005; Hass *et al.*, 2007; Metzdorff *et al.*, 2007; McPherson *et al.*, 2008; Prins & Korach, 2008; Chen *et al.*, 2009; Zhu & Imperato-McGinley, 2009; Walters *et al.*, 2010). Male reproductive organs such as the prostate express 5alpha-reductase and aromatase, and are thus capable of converting testosterone to AR ligand dihydrotestosterone (DHT) as well as to oestradiol (Zhu & Imperato-McGinley,

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2009; Ellem & Risbridger, 2010). DHT in turn can further be converted to ER beta ligand 3 beta-diol (Oliveira *et al.*, 2007).

A wide range of chemicals can disrupt male sexual differentiation by interfering with the physiological action of either androgens or estrogens during foetal and early postnatal life, by suppressing foetal androgen synthesis, blocking the androgen receptor or activating oestrogen receptors. The present investigation aims at elucidating the effect of environmentally relevant mixtures of endocrine active chemicals on male sexual differentiation.

Many chemicals with anti-androgenic action have been shown to act together in combination (Hass *et al.*, 2007; Metzdorff *et al.*, 2007; Howdeshell *et al.*, 2008; Rider *et al.*, 2008, 2010; Christiansen *et al.*, 2009). There is also experimental evidence that anti-androgens in combination can produce effects at doses that individually are not associated with any observable responses (Hass *et al.*, 2007; Howdeshell *et al.*, 2008; Christiansen *et al.*, 2009). Although the doses applied in these experiments were in the range of no-observed-adverse-effect-levels (NOAELs), they were still quite far removed from environmental exposures experienced by humans. Furthermore, these studies were designed primarily to explore the predictability of mixture effects using various assessment concepts, but not to investigate environmentally relevant combinations. Chemicals with oestrogenic action can also disturb the development of male reproductive organs (Kang *et al.*, 2002; Timms *et al.*, 2005; Durrer *et al.*, 2007; Prins *et al.*, 2008; Salian *et al.*, 2009; Axelstad *et al.*, 2011), but little is known about the effects of mixtures of oestrogenic and anti-androgenic chemicals.

Consequently, experimental evidence about the ways in which a wider range of endocrine disrupting chemicals behaves at doses approaching environmental levels has so far been missing. In the current study, for the first time, an in vivo rat experiment with mixtures of endocrine disrupting chemicals, modelled on information about environmental exposures, has been performed.

Considering the role of androgens and estrogens in sexual development, we selected chemicals known to exert anti-androgenic or oestrogenic actions. In composing the mixture to be assessed for endocrine disrupting effects in the rat, we encountered several difficulties. First, it was necessary to select chemicals where at least rudimentary information about their endocrine disrupting effects in vivo was available. It turned out that this demand already placed significant restrictions on the choice of possible candidate chemicals. Secondly, data about human exposures had to be available to guide the choice of doses to be combined in the mixture. However, reliable information about human exposures was patchy for certain chemicals, e.g. parabens and UV-filter substances.

On the basis of the available information, we selected 13 chemicals to be included in the test mixture. These were the phthalates di-*n*-butyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP), the pesticides vinclozolin, prochloraz, procymidone, linuron and epoxiconazole, the UV-filter substances octyl methoxycinnamate (OMC) and 4-methyl-benzylidene camphor (4-MBC), the pesticide metabolite p,p'-DDE, the phenolic compound bisphenol A(BPA), the preservative butyl paraben (BP) and the analgesic drug paracetamol (PM). Table 1 gives an overview of the uses of these chemicals, their presumed modes of action in causing reproductive tract alterations in experimental animals, some of the relevant effects seen in rodents, together with the NOAELs and lowest-observed-adverse-effect-levels (LOAELs) reported in the literature for these effects.

For the majority of the selected chemicals, disruption of male sexual differentiation in vivo by an anti-androgenic mode of action is quite well documented. One group of anti-androgens were phthalates. DBP (di-*n*-butyl phthalate), DEHP (di-(2-ethylhexyl) phthalate) and other phthalates are used to impart flexibility on plastic materials. They are known to exert demasculinizing effects by suppressing foetal testosterone synthesis during critical stages of sex differentiation in the rat where they induce malformations of internal sex organs and changes in anogenital distance (AGD) and nipple retention (NR) (Parks *et al.*, 2000; Schultz *et al.*, 2001; Wilson *et al.*, 2004; NRC 2008; Christiansen *et al.*, 2010). Vinclozolin is a widely used dicarboximide fungicide for the treatment of fruits, vegetables, ornamental plants and turf grasses (Kelce *et al.*, 1994; Kavlock & Cummings, 2005). Another group of anti-androgens were the pesticides. Vinclozolin metabolites compete with androgens for AR binding (Kelce *et al.*, 1994), suppress androgen-dependent gene transcription (Kelce *et al.*, 1997) and induce reproductive tract abnormalities including hypospadias, AGD changes and NR in rats (Gray *et al.*, 1994, 1999). The imidazole fungicide prochloraz is used for the preparation of soil for planting (Vinggaard *et al.*, 2005). Prochloraz is an AR antagonist and can interfere with testosterone synthesis by inhibiting the CYP450 17α-hydroxylase/17,20-lyase as shown in vitro studies (Vinggaard *et al.*, 2005, 2006). In the rat, it induces hypospadias, cryptorchidism and affects the development of several androgen-dependent tissues (Ostby *et al.*, 1999; Laier *et al.*, 2006). The dicarboximide fungicide procymidone is structurally related to vinclozolin; however, its potency as an anti-androgenic agent in vivo is approximately twofold lower (Ostby *et al.*, 1999). Procymidone is an AR antagonist and induces inhibitions of AR-mediated gene expression (Simard *et al.*, 1986; Ostby *et al.*, 1999; Wolf *et al.*, 1999; Hass *et al.*, 2007). In the rat, it induces an effect spectrum similar to that of

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Table 1 Features of the 13 chemicals selected for inclusion in a mixture

Chemical	Use	Mechanisms of action	Reproductive tract alterations	Effects on AGD & NR	NOAEL (mg/kg/day)	LOAEL (mg/kg/day)
DBP	Phthalate, used as plasticizers	Inhibitor of testosterone synthesis *(4, 10)*	Cryptorchidism and decreased sperm production *(5, 6)*	AGD ↓, nipples ↑ *(5, 6)*	50 *(22)*	100 *(22)*
DEHP	Phthalate, used as plasticizers	Inhibitor of testosterone synthesis *(4, 7)*	Cryptorchidism, testicular and epididymal abnormalities *(1, 2, 3, 4)*	AGD ↓, nipples ↑ *(2, 3, 4)*	3 *(2)*	10 (5.0 for NR) *(2)*
Vinclozolin	Dicarboximide fungicide	AR-antagonist *(12, 13)*	Cleft phallus with hypospadias, cryptorchidism, vaginal pouch *(12, 13, 14)*	AGD ↓, nipples ↑ *(13, 14, 23)*	5 *(23)*	10, 5.0 for NR *(23)*
Prochloraz	Imidazole fungicide	AR-antagonist, inhibitor of foetal steroidogenesis *(16)*, oestrogen receptor antagonist *(17)*	Reduction in testosterone, increase in progesterone levels in male rat foetuses at GD 21 in testis and plasma *(16, 17)*, mild dysgenesis of external genitalia *(17)*	AGD ↓, nipples ↑ *(15, 17)*	5 *(24)*	10 *(24)*
Procymidone	Dicarboximide fungicide	AR-antagonist *(3, 23, 25)*	Reduced weight of several anti-androgen-dependent tissues, hypospadias and cryptorchidism *(25)*	AGD ↓, nipples ↑ *(3, 23, 25)*	10 *(23)*	25 *(23)*
Linuron	Urea-based herbicide	AR-antagonist *(8, 9)*, inhibitor of foetal testosterone synthesis *(8)*	Enlarged testes, testicular and epididymal abnormalities *(11)*, reduced testicular testosterone production *(8)*	AGD ↓, nipples ↑ *(8, 11)*	25 *(26)*	50 *(26)*
Epoxiconazole	Triazole fungicide	The site of action is possible lyase function of CYP17 in steroidogenesis *(27)*	No reproductive tract malformations detected, marked fetotoxicity in dams who were unable to deliver *(27)*	no effects *(27)*	15 *(27)*	–
p,p′-DDE	Metabolite of the insecticide DDT	AR-antagonist *(29)*	hypospadias *(3)*	AGD ↓, nipples ↑ *(29, 30)*	-	10 *(30)*
4-MBC	UV-filter	Oestrogenic *(31)*, possess *in vitro* AR-antagonist potential (38)	Delay of preputial separation and weight decreases of ventral prostate *(33)*	-	0.7 *(33)*	7 *(33)*
OMC	UV-filter	Oestrogenic *(31)*, possess *in vitro* AR-antagonist potential (38)	Decreased testosterone levels and decreased relative weight of testis (g/100 g bw) at PND 16, reduced sperm count at 8 month of age *(34)*	-	-	500 *(34)*
Bisphenol A	Plasticizers	Oestrogenic *(35)*, *in vitro* AR antagonist (*39*)	Decreased sperm count and motility *(35)*	-	5 *(37)*	1.2 μg/kg/day –for effects seen in *(35)*
BP	Antifungal preservative in cosmetics	Oestrogenic *(36)*, *in vitro* AR Antagonist (*38*)	Decreased sperm count and motility *(36)*	-	-	100 *(36)*
Paracetamol	Analgesic and antipyretic	Inhibitor of prostaglandin synthesis *(19, 20)*	Positive association between ingestion during first and second trimester and cryptorchidism in humans *(19)*	AGD ↓, nipples ↑ *(18)*	-	350 (for AGD 150) *(18)*

AGD ↓ = decreased AGD, nipples ↑ = retained nipples. A hyphen (–) denotes cases where values could not be located in the open literature. References are bracketed italics numbers and listed in the footnotes to this table.

References: 1 = (Andrade *et al.*, 2006); 2 = (Christiansen *et al.*, 2010); 3 = (Wolf *et al.*, 1999); 4 = (Parks *et al.*, 2000); 5 = (NRC, 2008); 6 = (Mylchreest *et al.*, 1998); 7 = (Wilson *et al.*, 2004); 8 = (Hotchkiss *et al.*, 2004); 9 = (McIntyre *et al.*, 2001); 10 = (Schultz *et al.*, 2001); 11 = (McIntyre *et al.*, 2002); 12 = (Kelce *et al.*, 1994); 13 = (Gray *et al.*, 1999); 14 = (Gray *et al.*, 1994); 15 = (Vinggaard *et al.*, 2005); 16 = (Vinggaard *et al.*, 2006); 17 = (Laier *et al.*, 2006); 18 = (Hass *et al.*, 2010); 19 = (Kristensen *et al.*, 2011); 20 = (Kristensen *et al.*, 2010); 21 = (Buser *et al.*, 2006); 22 = (Mylchreest *et al.*, 2000); 23 = (Hass *et al.*, 2007); 24 = (Christiansen *et al.*, 2009); 25 = (Ostby *et al.*, 1999); 26 = (McIntyre *et al.*, 2001); 27 = (Taxvig *et al.*, 2007); 28 = (Scialli *et al.*, 2010); 29 = (Kelce *et al.*, 1995); 30 = (You *et al.*, 1998); 31 = (Schlumpf *et al.*, 2001); 32 = (Axelstad *et al.*, 2011); 33 = (Durrer *et al.*, 2007); 34 = (Cousins *et al.*, 2002); 35 = (Salian *et al.*, 2009); 36 = (Kang *et al.*, 2002); 37 = (European Food Safety Authority (EFSA) (2010)); 38 = (Ermler *et al.*, 2011) and 39 = (Ermler *et al.*, 2010).

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vinclozolin (Ostby *et al.*, 1999; Hass *et al.*, 2007). Linuron is a urea-based pre- and post-emergence herbicide applied to suppress broadleaf and grassy weeds. Linuron has been recognized as a weak AR antagonist (McIntyre *et al.*, 2002; Hotchkiss *et al.*, 2004) with a chemical structure quite similar to that of the AR-antagonist flutamide (McIntyre *et al.*, 2001). Linuron also affects foetal testis testosterone synthesis in a way similar to DBP (Hotchkiss *et al.*, 2004). Epoxiconazole is a triazole fungicide used for the control of fungi in horticultures. The mechanism of action of epoxiconazole is suggested to be at the lyase function of CYP 17 in the steroidogenesis (Taxvig *et al.*, 2007). The DDT metabolite p,p'-DDE continues to be relevant as an environmental pollutant because DDT is still manufactured in some countries, although it has been banned in the USA and in Europe (You *et al.*, 1998). DDE is an AR-antagonist (Kelce *et al.*, 1995) and causes hypospadias, altered AGD and NR in male rats after perinatal exposure (Kelce *et al.*, 1995; You *et al.*, 1998; Wolf *et al.*, 1999). One drug was included in the group of anti-androgens. Paracetamol, in the USA known as acetaminophen, is one of the most popular over-the-counter and prescribed analgesic and antipyretic medicine in the world (Ghanem *et al.*, 2009; Hass *et al.*, 2010). A recent Danish cohort study showed that intrauterine exposure (maternal intake during pregnancy) to mild analgesics (paracetamol) increased the risk of cryptorchidism among newborn boys and rat in vivo studies supported these anti-androgenic effects in humans (Jensen *et al.*, 2010; Kristensen *et al.*, 2011).

The oestrogenic chemicals contained in the mixture also affect male reproductive development, although the data in the literature are somehow less comprehensive.OMC and 4-MBC are frequently used as UV-filter substances in sunscreens and other cosmetics, and both have been found in human milk and blood samples (Hany & Nagel, 1995; Schlumpf *et al.*, 2010). OMC and 4-MBC are oestrogenic *in vitro* and in acute and 90-day in vivo models (Schlumpf *et al.*, 2001; Schreurs *et al.*, 2002; Tinwell *et al.*, 2002; Seidlova-Wuttke *et al.*, 2006). Pre- and postnatal administration of OMC caused reduced testosterone levels, relative testes and prostate weights and sperm counts in male offspring (Axelstad *et al.*, 2011). Pre- and postnatal exposure to 4-MBC delayed onset of puberty in male rat offspring, reduced prostate weight and, at higher doses, increased testis weight in adult offspring (Durrer *et al.*, 2007). Bisphenol A is used in the manufacture of polycarbonate plastic and is present in many consumer products. It can leach from the inner lining of tin cans and microwave containers into food during heating (Salian *et al.*, 2009), and in animal studies, exposure to BPA has been shown to cause decreased sperm counts and motility (Salian *et al.*, 2009) and affect the prostate (Timms *et al.*, 2005; Prins *et al.*, 2008). BP has anti-bacterial and anti-fungal properties, and is used as a food and cosmetic preservative. It is a well-recognized oestrogenic agent and has AR antagonist properties in vitro (Ermler *et al.*, 2010). BP has also been shown to reduce sperm counts and motility in rats (Kang *et al.*, 2002) All four selected estrogens have further been shown to antagonize the androgen receptor in vitro (Ermler *et al.*, 2010), but data on 4-MBC are conflicting (Ma *et al.*, 2003; Schreurs *et al.*, 2005). However, there are no in vivo reports showing typical anti-androgenic effects such as changes in AGD or NR for any of the substances. Both UV-filters and bisphenol A also interact with thyroid hormones (Moriyama *et al.*, 2002; Schmutzler *et al.*, 2004; Zoeller *et al.*, 2005); such effects may also interfere with developmental processes. Inclusion of these compounds offered the additional possibility of investigating if combined exposure to both anti-androgens and oestrogenic agents would magnify or counteract any adverse reproductive effects in the offspring.

In selecting the chemicals for the experimental mixture, we had to make compromises in terms of the number of chemicals that could be included, to keep the experiments manageable. Although we used high end human intake estimates to guide the choice of mixture ratio, we made adjustments to compensate for certain omissions. For example, we did not incorporate the phthalates DiBP, BBP and DINP and therefore adjusted the estimated high end intakes for DBP and DEHP upwards. These two phthalates now functioned as 'placeholders' to represent total exposure to phthalates with anti-androgenic activities. Similarly, epoxiconazole was used as a proxy for the entire group of anti-androgenic azole fungicides. For certain chemicals, we made downward adjustments to reflect the fact that only certain population groups are highly exposed. This was the case with BP and 4-MBC. Table 2 documents how the high intake estimates compared with the adjusted exposures that we used as a basis for setting the mixture ratio of the selected 13 compounds. The adjusted high end human exposures summed up to a value of 1.12 mg/kg/day.

Finally, we had to decide on the combined dosages that were to be used in the experimental study. We approached this by utilizing the so-called point of departure index (PODI), an application of the mixture assessment concept of dose addition:

$$PODI = \sum_{i=1}^{n} \frac{EL_i}{POD_i},$$

where *EL* denotes the estimated exposure level, and POD the Point of Departure which is a LOAEL, NOAEL or benchmark dose for a common endpoint (Wilkinson

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Table 2 Estimates for human intake of anti-androgenic chemicals and selection of dosages for the experimental mixture study

Chemical	High Intake (μg/kg d)	Adjusted intakes chosen as basis for mixture study (μg/kg d)	Comments and explanations
DBP	6	10	The high intake values are for a US population, based on the data communicated by Kohn *et al.* (2000) and NHANES, as collated by Benson (2009). The higher doses chosen as the basis for the mixture study reflect high exposures to other anti-androgenic phthalates
DEHP	3.6	20	
Vinclozolin	9	9	High intake is the life time Theoretical Maximum Daily Intake (TMDI) for France, as estimated by Menard *et al.* (2008)
Prochloraz	14	14	High intake is the life time TMDI for France (Menard *et al.*, 2008)
Procymidone	9	15	High intake is the life time TMDI for France (Menard *et al.*, 2008), this estimate was adjusted upwards to reflect exposure to other AR-antagonistic pesticides
Linuron	0.6	0.6	High intake is the life time TMDI for France (Menard *et al.*, 2008)
Epoxiconazole	1	10	Intake estimate by the European Standing Committee on the Food Chain and Animal Health, July 2008. The exposure chosen as a basis for the mixture study doses is intended to reflect high intakes of other azole pesticides. The combined life time TMDI (France) for all azole pesticides authorized for use in the European Union is around 5 μg/kg d (Menard *et al.*, 2008).
4-MBC	100	60	Based on intake estimates of the European Scientific Community on Consumer Products (SCCP) in their 2008 opinion on 4-MBC (SCCP, 2008)
OMC	100	120	Intakes were assumed to be similar to 4-MBC, based on the levels found in mother's milk. The higher doses chosen as the basis for the mixture study reflect that OMC is more frequently detected in human milk than 4-MBC (Schlumpf *et al.*, 2010).
p,p'-DDE	1	1	Estimates for a Polish population (Galassi *et al.*, 2008)
Bisphenol A	1.5	1.5	High intake estimate from EFSA (2006)
BP	100	60	The worst case systemic exposure dose was estimated to be 0.6 mg/kg/day by the European Cosmetic Toiletry and Perfumery Association (COLIPA), cited in VKM (2006). Cross & Roberts (2000) estimated a high intake of 0.1 mg/kg/day
Paracetamol	–	800	The dose in the mixture was chosen as around 10% of a pill a day to reflect the likely intermittent exposure.

et al., 2000). Assuming that all mixture components act together according to dose addition, significant combination effects are only expected to occur if the PODI is significantly greater than one. We calculated the PODI using NOAELs for anti-androgenic endpoints such as changes in AGD and NR. Where these were not available, LOAELs were used (p,p'-DDE and paracetamol). In view of the fact that 4-MBC, OMC, bisphenol A and BP are not known to affect AGD or NR, we assumed that these substances make no contribution to the anti-androgenic mixture effect. As shown in Table 3, the ratios of adjusted high exposure estimates (EL) and PODs summed up to a PODI of 0.016. This meant that a mixture dose of 1.12 mg/kg/day, equal to the sum of the adjusted high end human intakes, was not expected to produce any anti-androgenic effects in the rat. Even an increase of all individual doses by a factor of 62, where the PODI reaches 1, should not produce significant joint effects.

It is obvious that there are considerable uncertainties with these theoretical assumptions and calculations. We also had to consider the fact that experimental LOAELs determined in the listed studies exceed the corresponding NOAELs by a factor of two to three (see Table 1). Finally, the statistical power that was available with the chosen study design (see below) had to be taken into account. As a result of these considerations, it was our expectation that combined dosages 150 times higher than the adjusted human intakes in Table 2 (Mix150) should still be in the borderline range between a NOAEL and a LOAEL of the mixture, whereas dosages that are 450 times higher (Mix450) should lead to clear signs of disruption of sexual differentiation, with statistically significant changes in AGD and NR. Exclusion of the four estrogens 4-MBC, OMC, bisphenol A and BP from this mixture (AA-Mix450) should not diminish the overall effect.

We were conscious of the fact that the design of this exploratory mixture experiment had certain limitations. We used fewer animals than normally used in regulatory testing (14 instead of 20 per group) and, consequently, the statistical power was slightly reduced.

Furthermore, we did not test any of the single components in parallel with the mixture experiment, which prevented us from ascertaining their effectiveness after single administration. It was also not possible to conduct statistical dose-response regression analyses with the mixtures; as only two doses of the 13-component mixture

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Table 3 Dose selection for the experimental studies

Chemical	Adjusted intakes chosen as basis for mixture study (mg/kg/day)	Points of departure, POD (AGD, NR) (mg/kg/day)	Ratio of adjusted intakes to POD	Mixture dose (mg/kg/day)		
				Mix150	Mix450	Mix450A
DBP	0.01	50 [(1)]	0.0002	1.5	4.5	4.5
DEHP	0.02	3 [(1)]	0.0067	3	9	9
Vinclozolin	0.009	5 [(1)]	0.0018	1.35	4.05	4.05
Prochloraz	0.014	5 [(1)]	0.0028	2.1	6.3	6.3
Procymidone	0.015	10 [(1)]	0.0015	2.25	6.75	6.75
Linuron	0.0006	25 [(1)]	0.000024	0.09	0.27	0.27
Epoxiconazole	0.01	15 [(1)]	0.0007	1.5	4.5	4.5
4-MBC	0.06	– [(3)]	0	9	27	0
OMC	0.12	– [(3)]	0	18	54	0
p,p′-DDE	0.001	10[(2)]	0.0001	0.15	0.45	0.45
Bisphenol A	0.0015	– [(3)]	0	0.225	0.675	0
BP	0.06	– [(3)]	0	9	27	0
Paracetamol	0.8	350[(2)]	0.0023	120	360	360
Sum (mg/kg/day)	1.12			168	504	396
PODI			0.016			

Footnotes: [(1)] = NOAEL, [(2)] = LOAEL, see Table 1, [(3)] = because of lack of information about doses affecting AGD or NR.

(Mix150 and Mix450) and one dose of a mixture excluding 4-MBC, OMC, bisphenol A and BP (AAMix450) were tested.

Materials and methods

Test compounds

The test compounds in this study were DBP (purity >99.0%, CAS no. 84-74-2), DEHP (purity >99.5%, CAS no. 117-81-7), vinclozolin (purity >99.5%, CAS no. 50471-44-8), prochloraz (purity >98.5%, CAS no. 67747-09-5), procymidone (purity >99.5%, CAS no. 32809-16-8), linuron (purity >99.0%, CAS no. 330-55-2), epoxiconazole (purity >99.0%, CAS no. 106325-08-8), OMC (purity >98.0%, CAS no. 5466-77-3) and p,p'-DDE (purity >98.5%, CAS no.72-55-9), which were all purchased from VWR – Bie & Berntsen (Herlev, Denmark). The rest of the test compounds 4-MBC (purity >98.0%, CAS no. 36861-47-9), Bisphenol A (purity >99.5%, CAS no. 80-05-7), BP (purity > 99.0%, CAS no. 94-26-8) and paracetamol (purity > 99.0%, CAS no. 103-90-2), were all purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil, used both as a control compound and as a vehicle, was purchased from VWR – Bie & Berntsen.

Animals and exposure

Fifty-six time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day 3 (GD 3) of pregnancy. The study was performed using two blocks of 28 dams (separated by 1 week), and all dose groups were equally represented in the blocks, i.e. 14 mated rats per dose group were allocated. Dams that did not give birth were eliminated from the experiment.

The animals were housed in pairs until GD 17, and alone thereafter under standard conditions in semi-transparent polycarbonate type III cages (1291H Eurostandard Type III, Tecniplast) (15 × 27 × 43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Denmark) and plastic shelters (Brogaarden, Denmark). They were placed in an animal room with controlled environmental conditions with a 12 hour light–dark cycle with light intensity 500 lux starting at 9 pm, humidity 55% ± 5, temperature at 21 °C ± 1 °C and ventilation changing air 10 times per hour. All animals were fed on a standard diet with ALTROMIN 1314 (soy- and alfalfa-free, ALTROMIN GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in polycarbonate bottles (Tecniplast) was provided *ad libitum*.

On the day after arrival (GD 4), the time-mated dams were pseudo-randomly distributed into groups of 14 (6–8 per block) animals with similar body weight (BW) distributions. Mixtures and vehicle were administered by oral gavage with a stainless steel probe 1.2 × 80 mm (Scanbur, Karlslunde, Denmark) from GD 7 to the day before expected birth (GD 21) and again after birth from PND 1 to 22 to cover the most sensitive periods of the development of the reproductive system. However, PM was only added to the mixture groups at GD 13–19. The later initiation of PM exposure during pregnancy, i.e. GD 13

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instead of GD 7, was chosen to avoid potential effects on implantation of the embryos (Gupta *et al.*, 1981). The last dosing in this group during pregnancy was also earlier, i.e. GD 19 instead of GD 21, to avoid potential effects on the ability of the dams to give birth. The dams were not dosed with paracetamol in the mixture during lactation, because that would make the results more relevant in relation to our previous results based on only prenatal exposure (Kristensen *et al.*, 2011).

The animals were dosed by qualified animal technicians with vehicle (control, corn oil) or the three mixtures described in Table 3. All doses were given in vehicle (2 mL/kg) via oral gavage at the beginning of the animals' active period from 8 to 11 am. The solutions were prepared by a technician just before the study was performed as a stock solution, and during exposure period, they were stored in the animal rooms.

The dams were inspected twice a day for general toxicity including changes in clinical appearance (e.g. sedation and tremor). BWs were recorded on GD 4 and daily during the dosing period to monitor a decrease or increase in weight gain and to adjust dose according to weight.

We designated the day when a vaginal plug was detectable as gestation day (GD) 1 and the expected day of delivery, GD 23 as pup day (PD) 1.

The animal study was performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee.

Pregnancy and postnatal development

The weights of dams and individual pups were recorded after delivery in all the pregnant animals in the study. The pups were counted, sexed and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The BW of offspring was recorded on PD 6 and 13.

Anogenital distance and nipple retention

Anogenital distance (AGD) was measured in all the offspring at birth using an ocular stereomicroscope. On PD 13, all male and female pups were examined for the presence of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Female rats normally have 12–13 nipples while male rats normally have 0–1 nipples.

Section PD 16, male organ weights

The animals, 1 male pup per litter, $N = 11–13$ was weighed (bw) and decapitated. Testes, epididymides, ventral prostate, seminal vesicles, LABC (Levator ani/bulbocavernosus muscle), bulbouretral gland and liver were excised and weighed.

Statistics

For all analyses, the litter was the statistical unit. Data from continuous endpoints were examined for normal distribution and homogeneity of variance, and if relevant, transformed. AGD data were analysed by the AGD index, namely, AGD divided by the cube root of BW. The cube root transformation was used because it improves the comparison between the three-dimensional end point weight and the one-dimensional AGD (Gallavan *et al.*, 1999; Gray *et al.*, 1999). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. Organ weights were analysed using BW as a covariate, and maternal BWs were assessed with the number of offspring per litter as covariate. The number of nipple/areolas (NR) was assumed to follow a binomial distribution with a response range between 0 and θ_{max}, with θ_{max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θ_{max} was decided by considering the global fit (information criterion of Schwarz). Litter effects on NR and over-dispersion in the data were accounted using generalized estimating equations. Statistical significance were assessed using multiple contrast tests (Dunnett contrasts, global error rate $\alpha = 5\%$, two-sided) (Bretz *et al.*, 2005). These tests were chosen as they are already implemented in the SAS procedure PROC GENMOD which was used for all statistical analysis (SAS Institute Inc., Cary, NC, USA).

Results

There was no general maternal toxicity at any of the doses administered. The pregnancy proportion of the dams was 100%, which compares very favourably with the normal proportion of 80–90%. Consequently, the number of pregnant rats did not differ from the number of mated rats. One litter from the control group included only female pups, all other litters contained at least one pup from both genders.

The mixture exposures had no significant effect on the maternal weight gain during pregnancy (GD 7 to GD 21) or on the maternal weight on PND 1, and the mean gestation length was also unaffected (data not shown). Moreover, no changes in post-implantation loss, litter size, birth weights, sex ratio or perinatal loss were seen after *in utero* and lactational exposure to the different mixtures (data not shown).

The male offspring of dams treated with Mix150 already exhibited signs of anti-androgenic effects. The

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numbers of retained nipples were slightly elevated compared with untreated controls, and although the effect was small, it reached statistical significance, because the background occurrence in both untreated control groups was relatively low, even in comparison to previous studies in the same research facility (Table 4 and Fig. 1). At this mixture dose (168 mg/kg/day), the dosages of most individual components were considerably lower than their individual NOAELs or LOAELs. Changes in anogenital index were not observed with this mixture and the weights of liver, epididymis, seminal vesicle, LABC and bulbourethral gland were similar to untreated controls (Table 4). There were also no changes in female AGD, number of nipples or birth weights (data not shown). As anticipated on the basis of our PODI calculations, the male offspring of rats exposed to Mix450 were quite clearly affected by exposure. With an average number of nipples and areolas of 3.27, the effect was statistically significantly different from controls. There were no changes in male or female anogenital index. However, reductions in the weights of the ventral prostate and the left testis reached statistical significance, whereas right testis weights and pooled (left + right) testis weights could not be detected as significantly different from controls (Table 4).

Exposure to AAmix450, in which the four estrogens (4MBC, OMC, BPA and BP) were excluded, did not diminish the anti-androgenic effect in comparison to Mix450 exposure. The effect on NR even increased slightly, although this was not significantly different from the effect seen with Mix450 exposure (see Table 4 and Fig. 1).

The decreases in testes and prostate weights that were seen with Mix450 were, however, not observable with AAMix450 (Table 4). Interestingly, comparison of Mix450 with AAMix450 revealed a statistically significant increase in testis weights. This was seen for pooled testis weights as well as for left and right testis weights.

Discussion

Despite some uncertainties and knowledge gaps about the anti-androgenic potencies of the chosen chemicals, the PODI approach proved to be a surprisingly accurate tool for anticipating their combined effects on male rat offspring exposed during gestation and perinatally. For two compounds, p,p'-DDE and paracetamol, NOAELs were not accessible to us, but only their LOAELs were accessible, and consequently, we might have slightly underestimated the PODI. This uncertainty corroborated our expectation that doses 62-fold higher than the adjusted high end human exposure estimates should lead to no detectable disruption of sexual differentiation in the rat, and given our experimental design, we expected a 150-fold higher dose (Mix150) to be between a NOAEL and a

Table 4 AGD index, NR, body and organ weights from gestationally and perinatally exposed male rats

	PD0		PD13		PD16										
Mixture (dose in mg/kg/day)	Birth weight (g)	AGD index	BW (g)	No. of Nipples/ areolas	*N*	BW (g)	left testis (mg)	right testis (mg)	pooled testes (mg)	Epididymides (mg)	Ventral Prostate (mg)	Seminal vesicle (mg)	LABC (mg)	Bulbourethral gland (mg)	liver (mg)
Mixture of 13 compounds															
Control	6.27 ± 0.13	11.21 ± 0.12	27.17 ± 1.06	<0.1	11	32.41 ± 1.52	0.0540 ± 0.0017	0.0532 ± 0.0016	0.1071 ± 0.0033	0.0214 ± 0.0011	0.0114 ± 0.0007	0.0069 ± 0.0006	0.0245 ± 0.0017	0.0016 ± 0.0002	0.8097 ± 0.0476
Mix150 (168.12)	6.18 ± 0.11	11.09 ± 0.11	25.88 ± 0.89	**0.55** [b] [0.33–0.90]	12	31.00 ± 1.32	0.0531 ± 0.0022	0.0503 ± 0.0038	0.1033 ± 0.0058	0.0218 ± 0.0009	0.0098 ± 0.0008	0.0062 ± 0.0005	0.0263 ± 0.0012	0.0015 ± 0.0001	0.7986 ± 0.0407
Mix450 (504.50)	6.38 ± 0.09	11.04 ± 0.11	25.51 ± 0.62	**3.27** [b] [2.45–4.24]	13	30.70 ± 0.96	**0.0496** [a] ± 0.0014	0.0504 ± 0.0014	0.0999 ± 0.0027	0.0195 ± 0.0006	**0.0085** [a] ± 0.0006	0.0053 ± 0.0004	0.0226 ± 0.0010	0.0012 ± 0.0001	0.7691 ± 0.0289
Mixture of 9 anti-androgenic compounds															
AAMix450 (395.82)	6.36 ± 0.12	10.87 ± 0.11	26.14 ± 0.63	**4.16**[b] [3.57–4.80]	12	30.47 ± 0.73	0.0538 [c] ± 0.0018	0.0546 [c] ± 0.0016	0.1040 [c] ± 0.0048	0.0196 ± 0.0007	0.0089 ± 0.0005	0.0055 ± 0.0005	0.0217 ± 0.0009	0.0013 ± 0.0001	0.7650 ± 0.0232

Data shown are means ± SEM, and means with 95% confidence belts [in hard brackets]. $N = 14$ on PD 0 and PD 13.
[a], [b]Values statistically significantly different from controls are marked in bold ($p < 0.05$) and ($p < 0.01$).
[c]Values statistically significantly different from the 13-component mixture dose MIX450 ($p < 0.05$) are underlined.

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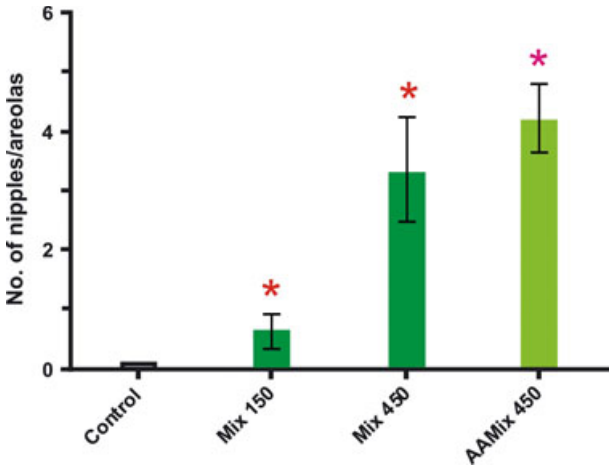

Figure 1 Effects of combined exposure to 13 and 9 endocrine disrupting chemicals on NR from perinatally exposed male rats on PND13. Results are shown as mean ± 95% confidence belt (N_{litter} = 13–14). Statistical significance is indicated by *, with $p < 0.01$. Mixture doses Mix150 and Mix450 refer to the 13-compound mixture, AAMix450 to the 9-compound mixture.

LOAEL for this mixture. As we found indications of anti-androgenic responses in terms of retained nipples at this mixture dose, our expectation was confirmed.

We had expected that Mix150 would be at the borderline between a NOAEL and a LOAEL, but found indications of anti-androgenic responses in terms of retained nipples. It appears that the exquisite sensitivity of this endpoint, combined with the low background occurrence in control animals made it possible that the effects became observable. Our experimental block-design lead to the testing of control animals in two independent blocks, and for both groups, we observed the same low incidence rate. Similar low incidences in control animals have been detected in our ongoing studies, and thus we rule out the possibility of singular control behaviour.

The results of our study suggest that highly exposed population groups, especially women of reproductive age, may not be protected sufficiently against the combined effects of chemicals that affect the hormonal milieu required for proper male sexual differentiation. Our PODI calculations indicated that doses 62-fold higher than the adjusted high end human exposure estimates should be tolerated by the rat without signs of disruption of sexual differentiation. As this exploratory experiment already allowed us to demonstrate effects with 150-fold higher doses, it is to be feared that an experiment with larger numbers of animals might have shown responses at even lower doses. This conjecture is speculative, but could be substantiated by a dose-response analysis with carefully spaced dosages. However, our study clearly shows that the PODI approach is very useful in formulating testable hypotheses to address that point.

Our concerns about insufficient margins of safety for highly exposed population groups have to be discussed critically in the context of the exposure estimates that were accessible to us, and in the light of the constraints that we faced when selecting the chemicals for our study.

The intake estimates we used for the chosen chemicals are based on those also employed by regulatory bodies; data of better quality are difficult to obtain or not available. Many of the intake estimates are based on maximum residue levels for the pesticides on food items and commodities, and can therefore be regarded as sufficiently conservative for the purpose of high intake estimates. However, the estimates for the UV-filter substances and BP relied on quite crude data. A notion implicit in our summing up of exposures/POD ratios to calculate the PODI is that certain populations might experience high exposures to all of the selected chemicals simultaneously. The case can be made that this is quite unlikely. To resolve this point, information about the co-occurrence of several chemicals in one and the same individual would be needed, but such data are not normally gathered in biomonitoring studies. It may be that more realistic data about simultaneous exposures to the selected 13 chemicals would have produced lower estimated high end intakes, with a lower estimated PODI and accordingly larger margins of safety.

However, these considerations need to be balanced against the constraints we had to deal with while selecting chemicals to be included in the mixtures. The knowledge gaps concerning the in vivo effects of other candidate compounds prevented us from including a wider range of chemicals in our mixtures. At least rudimentary information about the potency of chemicals in disrupting male sexual differentiation in vivo was required to safeguard against the risk of incorporating chemicals at too high doses. For example, there are more than 20 pesticides with in vitro AR antagonist properties in current use in the European Union (Kojima *et al.*, 2004; Vinggaard *et al.*, 2008; Orton *et al.*, 2011), but published in vivo data on NR and AGD are only available for a small number of these substances. Similar considerations apply to the large numbers of industrial chemicals and those used in consumer products that have also shown in vitro activity (Vinggaard *et al.*, 2008). Quantitative structure–activity relationship analyses have predicted that approximately 10% of the 30 000 chemicals listed in the European Inventory of Existing Commercial Chemical Substances display AR antagonism (Vinggaard *et al.*, 2008; Jensen *et al.*, 2011). Inevitably therefore our selection was biased, and cannot be regarded as being entirely representative of the spectrum of combined exposures

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encountered by human populations. It is likely that the inclusion of a wider range of chemicals would have led to more pronounced disrupting effects in our developmental toxicity model, with a larger PODI and correspondingly lower margins of safety.

The PODI approach assumes that the joint action of anti-androgens can be approximated by dose addition, and that antagonisms or synergisms are not relevant. That conjecture is supported by the available empirical evidence (Hass *et al.*, 2007; Howdeshell *et al.*, 2008; Rider *et al.*, 2008, 2010; Christiansen *et al.*, 2009). Antagonisms with mixtures of anti-androgens, which would lead to lower risk estimates, have not yet been reported. Of note is a synergism in relation to penile malformations in the rat that was observed with a mixture of anti-androgens with diverse modes of action (Christiansen *et al.*, 2009). However, in the same experiment, similar synergisms where not found with other anti-androgenic endpoints such changes in AGD or NR. As we applied the PODI method to these endpoints, it can be assumed with some plausibility that the premises of that approach are fulfilled.

Another aim of the study was to compare the effects of combined exposure to oestrogenic and anti-androgenic agents with exposures to anti-androgens alone. With regard to NR, an endpoint that is not affected by either one of the four included estrogens, we saw that adding oestrogens to the mixture did not enhance the observed endocrine effect. In fact, inclusion of the estrogens may even have led to small reductions of the effect. NR in the group without estrogens (AAMix450) was more pronounced than in the group including all 13 chemicals (Mix450), but the difference between these two groups was not statistically significant. With regard to postnatal prostate weight, the oestrogens alone can affect this endpoint, and it therefore fits well with the present results that a significant reduction was seen in animals exposed to all 13 of the compounds (Mix450), but not when only exposed to the nine anti-androgens (AAMix450). The reduction in right testes weight in the Mix450 group may have been a chance finding, as no effect on the left testes or the combined testes weights were seen. We are currently addressing these issues by assessing the effects of the oestrogenic chemicals in the absence of anti-androgens.

The PODI approach is helpful in identifying the chemicals that contribute most to the joint effect of the mixture, and could pinpoint substances that should be targeted for risk management measures, with high impact. As can be seen in Table 3, the intake estimate/POD ratios were particularly high for the phthalates, the dicarboximide and imidazole pesticides and paracetamol, which means that these substances are expected to have a large impact on the observed combined effects. However, it should be noted that human exposure to many of these agents is intermittent, and not continuous, as in our experiment. The average human exposures may therefore be lower, but if peak exposures during a vulnerable period are critical, as is suspected with anti-androgens, information about average exposures may be of limited value in predicting risks.

These considerations are relevant in the context of recent developments in Denmark where the Environmental Protection Agency has submitted a proposal to the European Chemicals Agency (ECHA) for restricting the four phthalates DEHP, BBP, DBP and DIBP. The proposal seeks to ban these phthalates in products for indoor use or in products with which consumers are in direct contact. This is the first proposal on restrictions to the ECHA on combined exposure data (Chemical Watch 2011; Milmo, 2011). Furthermore, in May 2011, the Danish Minister of the Environment announced the intention of introducing a Danish ban on the use of these four phthalates. She justified this with reference to alternative chemicals in use today which can readily replace phthalates in toys and children's products (Danish Ministry of the Environment 2011). As this is the first time that a ban is motivated by cocktail effects of chemicals, it may seem that the traditional focus of risk assessment on single chemicals is shifting towards considering combination effects. Our work shows that such efforts can be supported by using the PODI method, perhaps even without conducting time-consuming and costly animal experiments.

The present data demonstrates that exposure of the developing mammal to human high end mixture of oestrogenic and anti-androgenic chemicals can affect male sexual development. The investigation will be extended to include observations in both sexes and in reproductive organs as well as brain, during early stages of development and in adult offspring. As follow-up to this exploratory study, we are also currently carrying out studies aimed at exploring mixture effects of anti-androgenic and other chemicals at mixture doses around 150 times human high end exposures.

Significant efforts will be needed to close the gap between the increasing amount and diversity of chemical micro-pollutants and the available data for risk assessment, in particular, with respect to developmental exposure.

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Panel discussion

David Kristensen (Copenhagen, Denmark):
Did you measure the weight of the rats at post-natal day 13 to see if there was a difference between the exposed and unexposed groups?

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Ulla Hass (Søborg, Denmark):

There was no difference either in birth weight or in pup weight during lactation.

Philippe Grandjean (Odense, Denmark):

What is the mechanism of action on the effect of paracetamol in anogenital distance (AGD) and nipple retention? It is used extensively as an analgesic, but should we be concerned about its endocrine disrupter effect?

Ulla Hass:

I study effects but I have less understanding of the underlying mechanisms. We have previously found that prenatal exposure to paracetamol causes reduced AGD in male fetuses and now we have seen increased nipple retention in male rat pups. Clearly there is an anti androgenic action during pregnancy but I do not know the precise mechanisms. I think that pregnant women should be aware of the potential hazards and should only take paracetamol when absolutely necessary, and under medical advice.

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Effects of perinatal ethinyl estradiol exposure in male and female Wistar rats

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Keywords:
Endocrine disruption, estrogen, anogenital distance, nipples, mammary gland, gene expression, onset of puberty, genital malformations.

Abstract

Endocrine disrupting chemicals with estrogenic activity can affect offspring adversely after perinatal exposure. To evaluate estrogen-sensitive endpoints in offspring, time-mated Wistar rats (n=10) were gavaged during gestation and lactation with 0, 5, 15 or 50 μg/kg bw/day of ethinyl estradiol.

Increased anogenital distance, increased urethral slit length and number of nipples was observed in female offspring. Additionally, prostate weights and mRNA levels were affected prepubertally. For male and female mammary gland histology, prostate histology, timing of vaginal opening and estrous cyclicity, the expected differences were weak or absent, possibly due to the termination of exposure at weaning.

In conclusion, several parameters were affected by ethinyl estradiol in offspring before puberty, and following withdrawal of ethinyl estradiol exposure, this perinatal exposure gives rise to persistant genital malformations. Effects on female external sexual characteristics and changes in prostates and mammary glands should be in focus of future studies on estrogenic environmental chemicals.

Introduction

In humans, prenatal exposure to estrogenic chemicals such as diethylstilbestrol (DES), has been shown to adversely affect the reproductive development [1;2] and is furthermore associated with an increased risk of cancer in adulthood [1;3;4]. In toxicological *in vivo* studies, several endocrine disrupting compounds with an estrogenic mode of action have also been shown to adversely affect offspring after perinatal exposure. These include synthetic estrogens like ethinyl estradiol and 17β–estradiol, as well as phytoestrogens, butyl- and propylparaben (used as preservatives in cosmetics), bisphenol A (used in polycarbonate plastics), and some UV-filters (used in sunscreens and packaging) [5-10]. These are all compounds to which humans are exposed, and it is therefore important to obtain more knowledge on endpoints that in toxicological studies are sensitive to estrogenic action.

The present study aimed at exposing pregnant rat dams to ethinyl estradiol as a positive control compound for estrogenic chemicals. The chosen dose levels were expected to affect reproductive parameters while avoiding foetal/neonatal morbidity [11-14]. Previous studies examining the reproductive effects of ethinyl estradiol as a model for estrogenic activity have mostly used Sprague-Dawley and Long-Evans rats. The present study was conducted in outbred Wistar rats in order to investigate whether this rat strain is sensitive to a potent estrogen. Furthermore, some endpoints that have not previously been investigated thoroughly after perinatal ethinyl estradiol exposure, including malformations of female genitalia and changes in male mammary glands, were included in the present study.

Two commonly used endpoints in reproductive toxicity studies examining endocrine disrupting effects are anogenital distance (AGD) and nipple retention. These endpoints are very sensitive to antiandrogenic action in male offspring [15-17], whereas perinatal ethinyl estradiol exposure has not previously been shown to affect AGD or nipple retention in males [11;18]. In female offspring, AGDs have been shown to be affected by ethinyl estradiol, although findings are not consistent among studies. [19-24]. In the few studies investigating nipple retention in females, no significant effects of estrogenic compounds were seen [5;23].

Estrogenic compounds are known to enhance female mammary development and ethinyl estradiol has been shown to cause lobular hyperplasia in adult females [13;14]. Little is still known about effects of estrogenic chemicals on male mammary glands and conflicting findings have been found as some investigators describe a shift to a female-like morphology in male mammary glands after exposure to estrogenic compounds, while others do not [12;25-28]. Data on early mammary development were presented in Mandrup et al (2012) [29], and showed that ethinyl estradiol affected the density of mammary whole mounts at pup day (PD) 21-22 in both males and females, caused increased number of terminal end buds in males and increased outgrowth in females. In the present paper, elaborated examinations of female and

male mammary glands in whole mounts at PD50 and in histological sections at PD50 and 90 are presented, with the aim of determining whether examination of early mammary development by whole mount examination is a more or less sensitive marker of estrogenic influences than standard histological evaluation in adult animals.

Other reproductive parameters that have previously been shown to be sensitive to perinatal estrogen exposure include timing of sexual maturation, regularity of the estrous cycles, external genital malformations, male reproductive organ weight and prostate development. Several estrogenic compounds, including ethinyl estradiol, 17β-estradiol and bisphenol A have been show to advance puberty in female rats [23;30-32], whereas exposure to estrogenic compounds seems to delay sexual maturation in Sprague-Dawley and Long-Evans males [14;33-35]. Ethinyl estradiol, genistein and bisphenol A, have also been shown to cause more animals to cycle irregularly [22;36;37], and studies investigating the effects on external genital malformations in females have shown an increase in the urethral slit length in Sprague-Dawley rats exposed to ethinyl estradiol [24;38]. Prostate development is known to be affected by estrogenic compounds, as an increased incidence of prostatic lesions including hyperplasia and inflammatory cell infiltration has been shown in young adult rats after perinatal or neonatal exposure to estrogenic compounds [39;40]. These changes may be related to a decreased prostate weights seen before puberty [11] and altered expression of estrogen-sensitive genes. The current study therefore included histological examination of adult prostates and analysis of the mRNA expression for estrogen receptors (ER)α and ERβ and estrogen-regulated genes insulin-like growth factor 1 (IGF-1), ornithine decarboxylase (ODC) and Complement-C3 in the prepubertal prostate. As the prostate is profoundly regulated by androgen receptors, changes in mRNA levels of the androgen receptor (AR) and the AR regulated genes TRPM-2 and PBPC3 were also investigated in prepubertal prostates.

Reproductive hormones do not only organize development of the reproductive system but also affect neurological development. Since male activity levels have previously been shown to increase and approach female activity levels after postnatal estradiol exposure [41], assessment of this sex specific behaviour was investigated in the adult male and female offspring in the present study.

Collectively, the current study examined an array of endocrine-sensitive endpoints to examine the sensitivity of Wistar rats to the expected estrogenic effects previously investigated by others in Sprague-Dawley or Long-Evans rats, and to investigate further the influence of ethinyl estradiol on AGD, nipple retention and changes in mammary glands. The investigation was designed to investigate effects of ethinyl estradiol both following a period with full exposure of the dams to ethinyl estradiol (up to PD day 22) and a subsequent period where ethinyl estradiol was withdrawn (up to PD 90). This was done to investigate whether ethinyl estradiol leaves a footprint in the rats to cause (semi)permanent changes to rat physiology.

Materials and methods

Animals and study design

40 time-mated nulliparous female Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) arrived on gestation day (GD) 3. The day after arrival (GD4), the dams were assigned to four groups of 10 animals with similar weight distributions in all groups. The animals were housed in pairs until GD17 and alone thereafter until birth in semi-transparent polycarbonate cages (15x27x43 cm) with Aspen bedding (Tapvei, Brogaarden, Gentofte, Denmark). They were housed under the following controlled environmental conditions: 12h light/dark cycle with dark at 9AM to 9PM, 22°C ± 1°C, humidity of 55% ± 5 and 8 air changes per hour. A diet for growing rodents Altromin 1314 (soy- and alfalfa-free, Altromin GmBH, Lage Germany) and acidified tap water was provided *ad libitum*.

The dams were dosed by gavage from GD7 to the day before expected birth (GD21) and from pup day (PD) 1 to PD 22 with 0, 5, 15 or 50µg per kg body weight (bw) per day of ethinyl estradiol (CAS 57-63-6 from Steraloids, nr. E1550-000). From day 21 to PD 90 the offspring were not exposed to ethinyl estradiol. The control group was dosed with vehicle (corn oil, Sigma, Brøndby, Denmark, nr. C8267-2.5L). Independently of actual day of delivery, the expected day of delivery, GD23, was designated PD1. Thereby, the age of the pups was related to the time of conception, but was rather similar to postnatal age. The animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee of the National Food Institute at the Technical University of Denmark.

Evaluation of dams and offspring

Dams were distributed equally into four dose group on GD4, according to body weight, and body weight gain of the dams was registered daily from GD7 to GD21 and during lactation from the day after birth to PD22. Day of delivery was registered together with the weight and the distribution of male and female pups, and all live offspring were weighed again on PD6, 14, 22, 50/55, 76 and 90.

Evaluation of endocrine sensitive endpoints in live offspring after delivery

After delivery the weights of dams and individual pups were recorded and the pups were counted, sexed, checked for anomalies and anogenital distance (AGD) was measured using a stereomicroscope. Additionally, anogenital index (AGDI), i.e. AGD/cubic root of body weight was calculated for all offspring. Pups found dead were macroscopically investigated for changes when possible. On PD14, all male and female offspring were examined for the presence of areolas/nipples, as described in Jacobsen et al [42].

Necropsy PD21, 22 and 27

On PD21 one to two male pups per litter and on PD22 one to two female pups per litter were randomly selected for necropsy. The pups were decapitated in CO_2/O_2 anaesthesia. Liver, testis, ventral prostate, epididymis, seminal vesicle, levator ani/bulbocavernosus muscle (LABC) and bulbourethral glands, uterus were dissected from one male and one female per litter and weighed. Ovaries were dissected and weighed from 2 females per litter. Thyroids from female pups were excised from the thyroid cartilage and weighed, and thyroids from male pups were excised together with the thyroid cartilage and fixed in formalin for histological examination. Prostates were transferred to RNAlater for mRNA level analyses.

On PD27, two males and two females per litter were weaned for assessment of puberty onset, testing of motor activity levels and later sacrifice on PD50/55 and 90. The rest of the pups were sacrificed on PD22. Dams were anaesthetised in CO_2/O_2 and decapitated on PD27 and the number of implantations in the uterus was counted.

Onset of puberty

Onset of puberty was registered in all weaned male and female offspring. In female offspring sexual maturity was assessed by determining the day of vaginal opening (VO) as described by Goldman et al (2000) [43] and in male offspring puberty onset was assessed as time of preputial separation (PPS) [35;44]. Females were examined daily from PD27 and males from PD34. The age and weight at VO or PPS were recorded. Males were examined daily from PD34 until PPS was observed. On the day of VO or PPS the age and weight of the animals were recorded.

Motor activity levels

On PD57 all weaned offspring had their motor activity levels assessed during a 30 minutes test. At testing, the animals were placed individually in clean plastic cages without bedding, food or water, and the cages were placed in activity boxes with photocells which measured activity as described in Axelstad et al [45]. A computer automatically recorded output from photocells and collected data. Total activity during the 30 min observation period was used as a measure of general activity. In order to assess habituation, the 30 minute period was divided into shorter time intervals (3 x 10 minutes and 2 x 15 minutes) when analyzing the data.

Estrous cyclicity

Vaginal smears were collected from PD75 between 8 and 10 AM in the beginning of the dark period for the animals, vaginal cells were transferred to a glass slide using a moistened swab. The smears were fixed in 96% ethanol and stained with Gill's hematoxylin, Orange G6 and eosin-azure 50 (provided by VWR, Gentofte, Denmark) according to the adapted Papanicolaou (PAP stain) procedure reported by [46]. The stained smears were examined blindly to exposure group and stages were classified as Estrus (E), Metestrus (M), Diestrus (D) or Proestrus (P) or transitions between stages.

These stages were recognized by the presence, absence or proportional numbers of epithelial cells, cornified cells and leucocytes as described in OECD guidance document 106 and by Goldman and coworkers (2007) [47;48].

Necropsy PD50, 55 and 90

On PD50 (females), 55(males) and 90 one female and one male pup was anesthetised in CO_2/O_2 and decapitated. On PD50 vaginal smear was performed after sacrifice and stained with PAP stain to evaluate the specific stage of cycle. Ovaries and uterus were dissected and weighed and ovaries were fixed in formalin for histological examination. The uterus was weighed when intact as well as after draining. Abdominal (4^{th}) mammary glands from male and female pups were dissected for whole mount and fixed in formalin for histology.

On PD90 female pups were sacrificed in diestrous, evaluated by unstained vaginal smear. The vaginal smear was stained according to the same protocol for PAP staining and re-evaluated to confirm the stage of cycle. The following organs were excised and weighed: Liver, testis, epididymis, ventral prostate, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands, ovaries and uterus. The uterus was weighed when intact as well as after draining. Epididymis, ventral prostate, ovaries, uterus and 4^{th} mammary glands were fixed in formalin for histology. One testis was placed in Bouin's fixative for histological examination.

Genital malformations

Males were evaluated for testicular descent, external genital malformations and loss of hair as described by Christiansen et al (2008) [49] at necropsy on PD21, 55 and 90. On PD55 all live males were similarly evaluated for genital malformations.

Additionally, female pups were evaluated for malformations of the external genitalia at necropsy on PD22, 50 and 90. The urethral slit length was measured using a stereomicroscope with a scale (Figure 1), and a threshold for normal urethral slit length was set according to the deepest slit measured in controls.

Mammary gland whole mounts and histology

Whole mounts from PD50 females and PD55 males were scanned on a flatbed scanner (4800 dpi), and evaluation of the glands was performed using Image-Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). For male whole mounts PD55 the following parameters were measured: longitudinal growth, transverse growth, and the area (smallest polygon enclosing the mammary gland). The growth towards the lymph node was given a score (1-2 describing if the gland had reached the lymph nodes or not), and the density of the mammary glands was scored from 1 to 5 with 5 being a gland with high branching and budding. Female whole mounts PD50 were evaluated for the presence of beaded ducts as described by Vandenberg et al (2008) [50] and the density (score 1-5) in a standardised area between the nipple and the lymph nodes. The density scores for males and females were not comparable, as

the scoring criteria were adjusted for the normal gender differences at this age. Additionally, the development of lobules was evaluated in the female mammary glands PD50, both for predominant type of lobules present and the most developed type of lobule present in the gland (lobules type 1 to 3, as described by Russo et al 1988) [51].
Mammary glands for histology were routinely processed for paraffin embedding and stained with haematoxylin and eosin (H&E). At PD50, female mammary glands were evaluated in controls and in the highest dose group for lobule development (no lobular development, type 1 or type 2 lobules) and alveolar epithelium (single- or multi-layered). At PD90, female mammary glands were evaluated for lobuloalveolar pattern, lobule development, amount of fibrous tissue in the stroma, secretory material and dilation of ducts. Only mammary glands from females confirmed to be in diestrous or metestrous after staining of the smears and confirmed by histology of uteri and ovaries were used for statistical evaluation of the histological findings PD90. On PD50, female mammary glands were evaluated bearing the stage of estrous cycle in mind, as verified by stained smear and uterus histology. Male mammary glands PD55 were evaluated in controls and the highest dose group for vacuolation, secretory material in the ducts and tubuloalveolar pattern. Male mammary glands PD90 were evaluated in all dose groups for hypertrophy, epithelial vacuolisation and secretory material in the ducts. Additionally, male mammary glands were stained with periodic acid Schiff (PAS) and mucicarmine (Diagnostic BioSystems, Hague, Netherlands). PAS-stained mammary glands were evaluated for PAS positive secretory material and PAS positive granula in the cells. Mucicarmine stained mammary glands were evaluated for mucin positive secretory material.

Histology of reproductive organs and thyroids
Tissue samples for histological examination were routinely processed, embedded in paraffin, sectioned, stained with H&E and examined blinded to treatment groups.
Thyroids from male pups PD21 were examined.
Uteri and ovaries PD90 and uterus PD50 were evaluated for determination of stage of the estrous cycle. Ventral prostates PD90 were evaluated and were classified according to the dominating appearance of acini as regular (acini without or with minimal epithelial infoldings) or papillary (acini with folded epithelium) and according to the dominating epithelial type (simple squamous, cuboidal, columnar or high columnar). Additionally, the following were scored: Epithelial hyperplasia (score 0-4 depending on the precence of acini with areas of hyperplastic epithelium; no acini (0); few foci with ≤3 acini (1); multifocal foci with ≤3 acini (2); multifocal foci with > 3 acini (3); all acini (4)), epithelial atrophy (score 0-3 according to the proportion of acini lined by flattened epithelium), and interstitial, intraluminal and total inflammation (score 0-4 relative to severity of inflammation).
Adult testes were examined with emphasis on effects that may be related to endocrine disruption: a) spermatid retention, b) tubular dilation, c) degeneration of germ cells at specific stages, d) Leydig cell hyperplasia or adenoma. In the epididymal caput,

examination focused on a) presence of sloughed testicular cells in epididymal lumen, b) amount of spermatids c) vacuolization and degeneration in epithelium of main caput segment, d) disorganization of epithelium in initial segment, and e) interstitial inflammation.

Measurement of mRNA levels in prepubertal prostate

For mRNA level analyses, mRNA was isolated by use of the RNAeasy Mini Kit (Qiagen, Hilden, Germany) from samples stored in RNAlater. cDNA was next synthesised by use of the Omniscript RT kit (Life Technologies Europe BV, Naerum, Denmark) according to the description by the manufacturers. mRNA levels were then assessed by quantitative (q)PCR using TaqMan probes in combination with specific primer pairs. The investigated genes were: Insulin-like growth factor-1 (IGF-1), androgen receptor (AR), Transient receptor potential cation channel, Subfamily M, member 2 (TRPM-2), actin, Complement C3, Peroxisome proliferator-activated receptor (PPAR) α and γ, Eostrogen receptor (ER)-α and β, Ornithine decarboxylase (ODC), and Prostate specific binding protein polypeptide C3 (PBP C3) in the ventral prostate of PND 21 males. Furthermore, the housekeeping genes 18s rRNA and β-actin were evaluated. Primer and probe sequences are described in [52]. Primers and probes were mixed with TaqMan Fast Universal PCR Master Mix (Life Technologies Europe BV, Naerum, Denmark) and run on a Taqman 7900 HT qPCR apparatus (Applied Biosystems). The Ct value is the cycle number at which the amplified target reaches a defined threshold. First this value was determined for the raw qPCR data. Normalization was next done by subtracting the Ct value of the housekeeping gene 18s rRNA from the Ct value of the target gene. This is the delta Ct value (dCt). Then 2^{-dCt} values representing arbitrary values of mRNA copy numbers were used for graphs and statistical analysis to test for differences between treatment groups.

Statistics

For all statistical tests was used SAS Enterprise Guide 3.0 statistical software (SAS Institute Inc, Cary, NC, USA) or GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set at 0.05. Data with normal distribution and homogeneity of variance were analysed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA, or analysis were done on litter means. Body weight and number of pups in a litter was included as a covariate in analyses when relevant. A Dunnett post hoc test was used to correct for multiple comparisons. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall-Wallis test was used, followed by a Wilcoxon test for pair wise comparisons. Discrete data were analysed using a one-way analysis of variance (ANOVA) with body weight (bw) as a covariate.

The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and θmax, with θmax being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θmax was

decided by considering the global fit (information criterion of Schwarz). Litter effects on number of nipples and over-dispersion in the data were accounted by using Generalized Estimating Equations. Statistical significance were assessed using multiple contrast tests (Dunnett contrasts, global error rate $\alpha = 5\%$, two-sided) [53]. These tests were chosen as they are already implemented in the SAS procedure PROC GENMOD. Estrous cyclicity data were analysed using logistic regression and tested for over dispersion with Deviance and Pearson Goodness-of-Fit tests and correction for over dispersion due to litter effects were used when appropriate.
Histological data were analysed using a Fisher's exact test with two to four scores.
Regarding gene expression, -2dCt values were tested for normal distribution and analyzed by one-way ANOVA with post-test for linear slope and with Dunnett's test. For all genes the correlation between prostate weight and mRNA level was analyzed by use of Pearson correlation calculation (Graph Pad Prism).

Results

Reproductive parameters
The weight gain of dams from GD7 to GD21 was decreased in a dose-dependent manner with a statistically significant decrease at 50 µg/kg ($p<0.001$) (Table 1). Additionally, at 50 µg/kg, the gestation length was increased ($p<0.001$) (Table 1). There were no statistically significant effects on the number of implantations or perinatal loss of pups. No statistically significant difference in the distribution of females and males in the litters of dosed animals compared to controls was observed. At delivery, the weight of female pups was not affected. In contrast, the weight of male pups was decreased at delivery at 50 µg/kg ($p<0.05$).

AGD, nipple retention and body weight gain after delivery
In male offspring no statistically significant effects were observed in AGD or nipple retention. Female offspring from the high dose group had a significantly longer AGD and AGDI ($p<0.01$ for both) (Figure 2) and more nipples were present in offspring from group 4 (50 µg/kg) than in controls and this effect was statistically significant ($p<0.01$) (Figure 3).
After delivery, no effect on weight gain of dams was observed (Table 1). Body weights of offspring at necropsy are shown in table 2 and 3. In general, pups were smaller and had decreased growth when exposed to high doses of ethinyl estradiol. Growth in the exposed groups was decreased leading to smaller male pups PD6 at 50 µg/kg and at 15 and 50 µg/kg ethinyl estradiol when the animals reached PD14. Female pups were not statistically significantly smaller in any of the exposed groups at delivery, but growth was delayed leading to smaller pups in the highest dose-group at PD6, PD14 and PD22. On PD50-55 and PD90 no statistically significant differences were seen in the body weights.

Onset of puberty and estrous cycle determination

No statistically significant effects were observed on onset of puberty in male or female offspring compared to control. In females, 5 of 16 animals representing 3 litters in the high dose (50 µg/kg) were scored as already having developed VO on the first day of VO registration (PD27). At 15 µg/kg, 2 out of 18 animals, both belonging to the same litter, already showed VO on the first day of registration. No control animals or those receiving 5 µg/kg were scored as having VO on PD27.
No statistically significant effects were observed on estrous cyclicity although irregularly cycling animals were only observed in exposed groups. None of the 7 animals in the control group had irregular cycles, 2/8 (5 litters) in the group treated with 5 µg/kg, 3/10 (9 litters) in the group treated with 15 µg/kg and 2/8 (7 litters) in the highest exposure group, respectively.

Motor activity levels
No statistically significant effects on total motor activity levels or on habituation were seen after ethinyl estradiol exposure. Also, no significant differences between male and female activity levels were seen (data not shown).

Organ weights
At PD21, the ventral prostate weights were statistically significantly decreased in the group exposed to 15 µg/kg ($p<0.05$; Table 2). Interestingly, also other androgen-dependent male reproductive organs appeared smaller at 15 µg/kg than at 50µg/kg, although no statistically significant differences were observed (epididymides $p=0.07$, seminal vesicle $p=0.5$) (Table 2). No organs were weighed from males PD55. No statistically significant changes were found in organ weights of males PD90 (Table 3).
At PD22, weights of ovaries were statistically significantly decreased in the highest dose group ($p<0.05$) for both the ANOVA with body weight as a covariate and for relative ovary weights (Table 2) (data not shown for relative organ weights). On PD50 and 90, no changes in the weights of ovaries and uterus were found (Table 3). On PD90, one female in the lowest dose-group was distinctively smaller in terms of body weight than other females PD90 and was perceived as an outlier and thus removed from the dataset.

Genital malformations
In female pups, genital malformations were observed as a statistically significant increase in the urethral slit length was measured PD90 in females exposed to 50 µg/kg of ethinyl estradiol ($p<0.05$) (Figure 1). Similarly, on PD50 the mean urethral slit length showed a trend towards increased slit length, and a statistical significant increase was seen in the number of animals at 50 µg/kg showing larger clefts (long slit length) than controls ($p<0.05$) (Figure 1). No changes were observed in the urethral slit length on PD22. Upon examination of the female genitalia on PD22, two pups from the same litter in the highest dose-group showed starting development of VO. The same animals had an enlarged uterus with a markedly increased uterus weight compared to the rest of the female pups.

No genital malformations were observed in males at necropsy on PD21 or 90. On PD55, examination of live males showed two animals, one from each of the two highest dose groups, with unilateral cryptorchidism. At necropsy on PD55, three animals (one at 15 µg/kg and two at 50µg/kg) were found to have a mildly split penis and one animal had unilateral cryptorchidism. There were no statistically significant differences between groups.

Mammary glands

Overall, females PD50 were mainly presenting no lobular development or type 1 lobules as the predominant lobule development in the whole mounts of mammary glands. However, an increase in the type of the most developed lobule was observed. All but one control animal had lobules type 1 as the most developed type of lobule. One of 7 control animals, 2 out of 8 in each of the lowest dose-groups and 4 of 8 females from the highest dose group had lobules type 2 as the most developed lobules present (Figure 4A). However, this apparent shift from lobule type 1 to lobule type 2 as the most developed type of lobule in the high dose group was not statistically significant. No beads were found in the ducts of the mammary glands in the whole mounts. One control female PD50 had prolonged diestrous, thus showing different morphology in both whole mounts and histology and was not included in the statistics and the data presented.

Histological examination of controls and high-dose female mammary glands PD50 showed a higher frequency of high-dose females with multi-layered alveolar epithelium, however, this was not statistically significant (Figure 4B). Female mammary glands PD90 were evaluated, but statistics were restricted to females in diestrous or metestrous. Few animals were in another estrous stage (2 controls, 2 low dose, 0 middle dose and 3 high dose animals). All 10 females in the 15 µg/kg dose group were in diestrous. The evaluation of the female mammary histology PD90 showed no difference in the number of animals with regard to lobular development (lobules type 1 or 2) (data not shown). The number of dilated ducts appeared increased in the 15 µg/kg dose group, but this was not statistically significant (Figure 4C). No treatment-related differences were found in the distribution or the amounts of secretory material in the mammary glands and the dilated ducts were not associated with secretion of the mammary glands.

Mammary gland whole mounts from high dose male offspring PD55 showed no statistically significant changes in the longitudinal growth, transverse growth, area or density. However, a trend to an increased number of animals with mammary glands reaching the lymph node was observed (Figure 5A) and an increase in the number of animals with a high density score (score 5) was observed at 50 µg/kg (data not shown). Yet, no changes in male whole mounts were statistically significant. Due to technical difficulties in sectioning the mammary glands for histology, the number of controls PD55 was reduced to 5 and trends for histological changes were difficult to

interpret. Consequently, data from histology of male mammary glands PD55 were omitted.
On PD90 the number of males with hypertrophic mammary epithelium appeared increased (Figure 5B), but this was not statistically significant. No changes in tubuloalveolar pattern, vacuolisation of mammary epithelium or distribution of secretion filled ducts in the glands were observed (data not shown). PAS positive granula were found more often and were more abundant in the highest dose group compared with controls, however, no statistically significant differences between the groups was found for mucin-positive or PAS-positive secretion or granula (data not shown).

Histology of reproductive organs and thyroid gland
Histological examination showed no effects of dosing in male thyroid glands PD21 or testes and epididymides PD 90. Moreover, ventral prostates showed no statistically significant differences, although an increased prevalence of epithelial hyperplasia score 3, papillary epithelial infoldings and high columnar epithelium was observed at 5 and 15 µg/kg (Table 4). Histological appearance of prostates of the high dose group was comparable to controls. Interstitial and intraluminal infiltration with predominantly mononuclear inflammatory cells was found in all groups, but moderately severe inflammation was only seen in groups exposed to ethinyl estradiol (Table 4). Reactive hyperplasia was observed in prostates from all males with moderately severe inflammation (Table 4).

The stage of estrous cycle of females at necropsy on PD50 and PD90 was confirmed by vaginal smear and histologic examination of uterus PD50 and for PD90 with histology of uterus and ovaries. One control PD50 and one female in the highest dose-group PD90 were acyclic and were omitted from the data of mammary glands.

Gene expression
For prostate isolated on PD21, ODC and actin mRNA levels were increased by ethinyl estradiol as evaluated by post test for linear trend in connection with ANOVA (Figure 6). Regarding mRNA levels, there were no effects of ethinyl estradiol on AR, TRPM2, IGF-1, complement C3, PPARα, PPARγ, ERα ($p=0.06$ by post test for linear trend) and β, or PBPC3. By correlation analysis it was found that the prostate weight in the individual animal was correlated to the mRNA level of PBPC3 ($p<0.01$, data not shown).

Discussion
Several studies have examined reproductive effects of ethinyl estradiol in Sprague-Dawley and Long-Evans rats, and in the current study we examined effects in Wistar rats and included endpoints that have not previously been thoroughly investigated. This study reveals developmental effects in female offspring, in which an increased

AGD, an increased number of nipples, and an increased urethral slit length were observed. In males, perinatal exposure to ethinyl estradiol seemed to affect mainly the mammary glands and the prostates.

Effects on dams and pup growth

Pregnant dams and growth of pups were affected by ethinyl estradiol. The lower maternal weight gain during gestation in high dose dams is in accordance with findings in other studies with perinatal exposure to ethinyl estradiol [11;13;14]. Additionally, gestational length was increased in the highest dose-group, and to our knowledge this has not been reported for ethinyl estradiol before [22;23;38]. However, a prolonged gestational length has been reported for dietylstilbestrol (DES) [54;55].

In general, pup weight gain from delivery and onwards was decreased at 50µg/kg while dosing was on-going, confirming several other studies [13;14;24]. However, at weaning, this effect was smaller and only apparent in female offspring. This delayed growth of offspring may reflect a decreased lactation in high-dose dams especially affecting the offspring in the earliest period of lactation when supplementary intake of other food is absent.

Changes in female external sexual characteristics

Female pups exposed perinatally to ethinyl estradiol showed an increased, an increased AGD and AGDi, an increased number of nipples and an increased urethral slit length; effects that are not thoroughly investigated in the literature or are not consistent among studies.

The observed increased AGD in females treated with 50 µg/kg ethinyl estradiol compared with controls is in agreement with results by Ryan et al (2010), who found an increased AGD of female Long Evans rats at the same dose level and dosing period (GD7 to PND18) [23] and as reported in several other studies [19-22]. However, one study with similar exposure and dosing period found no effects of treatment in Sprague-Dawley rats [24].

There are only few reports describing effects on the number of nipples in females in the open literature, however, not with estrogenic exposure. Ryan et al (2010) did not observe increased number of nipples in female offspring in Long-Evans rats following ethinyl estradiol exposure to comparable doses and dosing period as in the present study [23]. Yet, in the National Toxicology Program (NTP) study hypertrophy of the nipples was found as an effect of ethinyl estradiol exposure [22]. If the observed increased number of nipples in females of the present study is caused by ethinyl estradiol exposure it is possible that excessive presence of estrogen somehow induces a higher number of mammary buds along the mammary streak during mammary development. It may also be speculated that some regression of nipple anlagen happens in females as well as in the males, a process which is then counteracted by ethinyl estradiol.

Females exposed to 50 µg/kg ethinyl estradiol had increased urethral slit length at PD50 and PD90, and this is in accordance with Sawaki et al showing an increase in

the urethral slit length and an increased number of adult animals having a deep urethral slit after perinatal exposure to 50 µg/kg ethinyl estradiol [24;38]. Thus, it was confirmed that the potent estrogenic compound, ethinyl estradiol, gives rise to genital malformations in female offspring after perinatal exposure.
Overall, ethinyl estradiol seemed to affect female external genital cheracteristics as seen as a change in both the number of nipples and changes in the genitals. The female external genitals were changes in young (increased AGD PD1) as well as adults (increased uthral slit length PD50 and 90), suggesting effects on the genitals leading to presistant changes present after withdrawal of the exposure.

Mammary glands
In adult mammary glands, no statistically significant changes were found. However, our previous study on prepubertal offspring from the same litters showed advanced development of mammary glands [29]. Whole mounts of mammary glands PD21-22 showed an enhanced development of female and male mammary glands represented by statistically significant differences in the outgrowth, density and number of terminal end buds [29]. Thus, prepubertal mammary glands seem to be more markedly affected by ethinyl estradiol compared to adult mammary glands, which could be interpreted as transient effects in prepubertal animals, but could also reflect low power for detection of persistent effects in adults.

In adult female mammary glands, a trend to increased lobular development was observed, indicating advanced differentiation, and although this was not statistically significant these trends mayreflect persistent effects of ethinyl estradiol on mammary glands. Changes in both female and male adult mammary glands was expected as lobular development (lobular hyperplasia) and increased secretory activity in females and feminization of male mammary glands have been described previously [13;14;26]. Takagi et al (2004) found increased lobular hyperplasia in adult female mammary glands after perinatal exposure to ethinyl estradiol and Murrill and co-workers (1996) observed a significant increase in lobules type 2 in 50 days old females exposed postnatally to genistein [14;56]. Dilated ducts were observed in ethinyl estradiol exposed females in the present study at PD90, but this did not correlate to an increased secretory activity of the mammary glands of exposed adult females. However, Biegel et al (1998) found an increase in the secretory dilation of alveoli in adult females after perinatal exposure to 17β-estradiol [26]. Yet, in that study, pups were exposed continuously through the diet until the day of necropsy and the same changes were found in adults exposed exclusively in adulthood until necropsy. Hence, increased secretory activity may be expected in females exposed to estrogens in adulthood, but may not be found in adults after exposure early in life.

No sign of feminization of the male mammary glands were present in this study as no increase in secretion or changes in tubuloalveolar pattern was observed. Biegel et al (1998) found an increase in tubuloalveolar morphology in adult male mammary glands after postnatal exposure to 10 and 50 ppm (50 ppm approximately equal to 2,5

µg/kg) of 17β-estradiol [26]. However, Latendresse et al (2009) did not observe feminization with tubuloalveolar growth of the adult male mammary glands after continuous exposure to 50 ppb ethinyl estradiol (approximately equal to 5 µg/kg) from conception until adulthood [12]. Thus, perinatal exposure to estrogenic chemicals may not always induce feminization of male mammary glands by developing a tubuloalveolar pattern.
However, other trends were observed. Male mammary glands had a tendency to become larger with dosing as was seen in whole mounts as an increased number of animals with mammary glands reaching the lymph node at PD55. Such a change could be expected, as estrogen is known to be responsible for ductal outgrowth [57;58]. Additionally, histology showed a tendency towards a higher prevalence of adult males with hypertrophic mammary epithelium. Consistently with these results, a study by Delclos et al (2001) observed increased hypertrophy of male mammary glands PD50 exposed perinatally and after weaning to genistein [59]. However, in the present study hypertrophy was present in 50% of controls and no difference between the dosed group and controls could be detected. It may be speculated that the lack of clear effects on this endpoint could be due to the termination of dosing at weaning in the present study.

Reproductive effects in female offspring
In the current study, 11% (2/18) and 31% (5/16) of female offspring exposed to the two highest doses of ethinyl estradiol were in puberty at the first day of registration, yet no statistically significant effects were seen on the weight or age at onset of puberty. Other studies have reported an earlier age of sexual maturation following perinatal exposure to ethinyl estradiol [22;23], and an earlier VO was also expected in this study. It is unknown at what age the few animals in puberty at the first day of registration had attained VO, and the registration of VO from PD27 may thus result in a weaker statistical result. Another reason for the lack of statistical significance is group size, as a report from ILSI 1999 estimated that a group size of 20 litters should be used when measuring effects on PPS and VO [60]. In comparison, our group size was 5-9 litters.

No statistically significant effects on estrous cyclicity were observed. However, irregularly cycling females were observed only in ethinyl estradiol exposed groups (7 of 26 exposed females). Others have shown an increased length of cycles and an increased number of abnormal cycles in rats exposed to a dietary dose of 50 ppb (about 5 µg/kg) of ethinyl estradiol, while lower doses (2 or 10 ppb) caused increased cycle length [22]. Other studies also using perinatal and continued exposure in adulthood by either oral gavage or dietary exposure showed similar effects characterized by persistent estrous [61], [20]. In the present study, the lack of significant effects may be due to the short dosing period terminating at weaning in contrast to the studies reported in the literature.

Effects in male offspring

Changes in prepubertal prostate weights and gene expression were the only male endpoints investigated in the present study that were significantly affected by perinatal ethinyl estradiol exposure. In prepubertal males, ethinyl estradiol reduced prostate weight at 15 µg/kg, but a normal prostate weight was seen at 50 µg/kg. More marked effects on male reproductive organ weights have been observed in other studies on perinatal exposure to ethinyl estradiol. Statistically significant reductions of testes and seminal vesicle weights and slight reduction of prostate weights were seen at 5 µg/kg, and statistically significant reduction of prostate, LABC and glans penis weights were seen at 50 µg/kg of ethinyl estradiol in adult Long-Evans rats exposed from GD7 to PND18 [11]. Although mean weights of testes, epididymides, seminal vesicle, bulboutrethal gland and LABC did appear reduced in the current study, these changes were not statistically significant and were only seen in prepubertal males.

Estrogenic compounds may alter expression of ER responsive genes such as IGF-1 [62], Compl C3 or ODC. In addition, reduced ERα mRNA and protein levels have been found in adult rat prostates following perinatal soy exposure [63]. The current study showed an increased mRNA level of ODC in ventral prostates of prepubertal rats. In addition, ethinyl estradiol was found to increase actin mRNA levels. Actin has previously been described to increase in rat uterus upon estrogen exposure [64], and other lines ef evidence suggest a role for estradiol in the control of the actin skeleton supporting that actin is an estradiol regulated protein [65]. Prostate weight was correlated to the PBPC3 mRNA level. Such a correlation has been described previously for flutamide [66]. Thus, the current data support that the weight of the prostate is important for the expression of this gene or vice versa.

These early changes in prostate weight and gene expression induced by ethinyl estradiol appear to be followed by persistent effects on prostate histology. Howdeshell et al (2008) observed ventral prostate hyperplasia PND150 in 2 of 29 rats at 5 µg/kg ethinyl estradiol and in 11 of 24 rats at 50 µg/kg ethinyl estradiol after perinatal exposure, while this was not seen in 31 control rats [11]. Likewise, an increased incidence of prostatic lesions including hyperplasia and inflammatory cell infiltration has been shown in young adult rats after perinatal or neonatal exposure to other estrogenic compounds [39;40]. In the current study more prominent epithelial hyperplasia was indicated in ventral prostate in young adults in the low and middle dose groups and moderately severe inflammation was observed in all dosed groups, but not in controls. The lack of hyperplastic response in the high dose group cannot readily be explained. However, other studies with perinatal dietary exposure of Sprague Dawley rats to 0.5 ppm (27-63 µg/kg) ethinyl estradiol did not show significant histopathological changes in prostate in young adults [13;14]. Although not consistently reported [67] non-monotonous dose-response patterns on prostate development have been described for natural and synthetic estrogens administered prenatally or neonatally to mice and rats [68;69].

No treatment related effects were observed on male AGD and nipple retention in the present study. Other studies in Long Evans and Sprague-Dawley rats perinatally exposed to ethinyl estradiol have reported no effects on male AGD at doses comparable to ours [11;13;14;18].

Unexpectedly, no significant effects were seen on the weight or timing of onset of puberty in the male offspring. Several studies report a delayed PPS following exposure to estrogenic compounds. PPS was delayed in male Sprague-Dawley rats perinatally exposed to 0.5 ppm ethinyl estradiol (27-63 µg/kg) in the diet [13;14] and developmental exposure to other estrogenic compounds has been reported to delay puberty in male rats [8;33;70]. However, no consistent effects were reported on PPS in male offspring in studies conducted on dietary exposure to ethinyl estradiol by the NTP. Delayed PPS was seen in the multi-generation study in the group receiving 50 ppb (approximately 4 ug/kg) but only in the F2 generation. In contrast, accelerated PPS was observed in the groups treated with 5 and 25 ppb (approximately 1.1 and 5.5 µg/kg , respectively) but not at higher doses in their range-finding study [22]. The lack of effects on PPS in the current study may be due to the short dosing period compared to other studies.

Motor activity levels

Male activity levels have previously been shown to increase after 17β-estradiol exposure on PND 3 and 5 [41], but no statistically significant effects were seen in motor activity levels in either male or female offspring in the present study. Usually a sex difference is seen for motor activity levels, with adult females exhibiting higher activity levels than adult males, irrespective of the rat strain used [5;23;41], however, in this study no statistically significant gender differences were seen in control animals. This may be explained by three females registered to have unusually low activity levels. Hence, based on the present study, it is not possible to conclude on the absence or presence of effect of ethinyl estradiol on sexual differentiation of the brain.

Sensitivity differences for examined endpoints

Several classical estrogen-sensitive endpoints such as sexual maturation, estrous cyclicity and mammary gland histology are evaluated as score measures and thus, the statistical power is low and may result in false negative conclusions compared to quantitative endpoints (e.g. organ weights and gene expression). Hence, a large number of animals is needed to see a difference between the dosed groups and the controls for many of the endpoints evaluated, even in the present case of a potent estrogenic chemical. Thus, for such endpoints, it is important to pay attention to trends and findings that may suggest an effect of exposure to the test material, as stressed in OECD guidance documents for histological evaluation [71].

As noted, other studies have shown statistically significant effects of estrogenic chemicals in these endpoints. For some endpoints, other studies with continued exposure appeared to show more marked effects than this study with exposure until

weaning. For other endpoints, variations in susceptibility of different rat strains is known, as e.g. SD rats are more susceptible to develop spontaneous and carcinogen induced mammary tumours compared to other rat strains as reviewed by Rudmann et al [72]. Likewise, most studies on e.g. mammary gland morphology and histology is, to our knowledge, studied in Sprague-Dawley or Long-Evans rats, and more knowledge on estrogen sensitive endpoints in Wistar rats is important for evaluation of e.g. regulatory toxicological studies, which are often performed in this rat strain.
AGD and the number of nipples are not score measures and are evaluated in a large number of animals (i.e. all offspring) and were shown to be sensitive endpoints for the effects of ethinyl estradiol in developing females. Similarly, future development of sensitive and preferably quantitative measurements to supplement the currently used endpoints known to be affected by estrogens, e.g. mammary gland morphology, may facilitate the detection of adverse effects of estrogenic compounds and concomitantly reduce the present need for a high number of experimental animals in toxicological studies.

Conclusions

The present study investigated endocrine-sensitive endpoints in male and female Wistar rats following perinatal ethinyl estradiol exposure. This potent estrogenic compound was found to induce an increased number of nipples in female offspring and an increase in malformations of external female genitalia, which seemed to be permanently affected after withdrawal of exposure. Malformations of female genitals were found in young as well as adult offspring appearing as an increased AGD at birth and a deeper urethral slit length in adulthood. In prepubertal male offspring, a decreased ventral prostate weight was seen at 15 µg/kg and estrogen-regulated gene expression was increased dose-dependently. Changes in mammary gland histology, timing of sexual maturation and estrous cyclicity were not statistically significant, but showed similar trends as observed in Sprague-Dawley and Long-Evans rats. The lack of clear effects on these endpoints may be due to the short dosing period terminating at weaning in contrast to other studies with continued exposures after weaning.
We conclude that perinatal ethinyl estradiol exposure of Wistar rats predominantly affect the morphology of the female reproductive system and prostate endpoints in males. Prepubertal male and female mammary glands also revealed marked changes and these endpoints should be in focus of future studies on estrogenic environmental chemicals, as potential chemical perturbation of mammary, prostate and female reproductive organ development during fetal life may have severe consequences later in life. Moreover, continued exposure after weaning should be considered in toxicological testing of potential estrogenic compounds, and future development of sensitive and preferably quantitative endpoints is encouraged.

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Table 1. Pregnancy and litter data. Data represent group means based on litter means ± SD. *p<0.05, ** p<0.01, ***p<0.001. AGD is analyzed with birth weight as a covariate. # male birth weight in group 3 seems to be decreased but it is not significant when bw and number af pups per litter as covariates in the statistical analysis. EE2: ethinyl estradiol.

	1: Control	2:EE2 5 µg/kg/bw	3:EE2 15 µg/kg/bw	4: EE2 50 µg/kg/bw
No. Of dams (litters)	10 (8)	10 (6)	10 (9)	10 (8)
Maternal weight gain GD7-GD21	70.4 ± 7.8	64.8 ± 14.7	65.7 ± 12	**41.0 ± 16.5*** **
Maternal weight gain GD7-PD1	14.2 ± 7.7	13.6 ± 5.9	9.4 ± 8.6	**-3.7 ± 6.3 *** **
Maternal weight gain PD1-22	4.6 ± 10	1.7 ± 11.7	1.6 ± 10.3	7.6 ± 7.5
Gestation length (days)	23 ± 0.0	23.2 ± 0.41	23 ± 0.0	**23.7 ± 0.46*** **
% post-implantation loss	2.2 ± 4.3	8.2 ± 7.5	4.5 ± 9.2	2.1 ± 5.9
% perinatal loss	8.0 ± 8.6	11.0 ± 6.9	7.9 ± 13.0	5.7 ± 12.0
Born alive per. Litter	10.4 ± 1.9	9.7 ± 2.9	11.1 ± 2.7	9.1 ± 3.9
% Postnatal death	8.4 ± 9.5	8.9 ± 9.0	3.4 ± 4.9	4.1 ± 7.8
% Males	57.6 ± 14.0	48.3 ± 18.9	48.5 ± 16.8	53.0 ± 17.0
Birth weight male offspring	6.2 ± 0.3	6.4 ± 0.4	6.0 ± 0.2#	**6.0 ± 0.5***
Birth weight female offspring	5.9 ± 0.3	5.9 ± 0.3	5.6 ± 0.2	5.7 ± 0.5
AGD males	21.3 ± 0.7	21.5 ± 0.7	21.2 ± 0.8	21.9 ± 0.8
AGD females	10.9 ± 0.7	11.0 ± 0.5	11.1 ± 0.3	**11.9 ± 0.9****
AGDi males	11.6 ± 0.5	11.6 ± 0.3	11.7 ± 0.4	12.1 ± 0.4
AGDi females	6.1 ± 0.4	6.1 ± 0.4	6.3 ± 0.2	**6.7 ± 0.3*****
Males bw PD6	13.0 ± 1.2	12.6 ± 1.	12.0 ± 1.2	**10.9 ± 1.1*****
Females bw PD6	12.5 ± 1.0	11.7 ± 1.1	11.6 ± 1.1	**10.5 ± 1.1*****
Males bw PD14	27.6 ± 2.6	26.2 ± 2.8	**24.8 ± 3.0***	**23.5 ± 3.2*****
Females bw PD14	26.4 ± 2.2	24.6 ± 1.9	24.2 ± 2.9	**22.8 ± 2.8*****
Males bw PD21	39.6 ± 5.1	39.2 ± 4.9	36.8 ± 5.1	**36.1 ± 5.9***
Females bw PD22	42.4 ± 3.5	38.8 ± 3.1	39.1 ± 5.7	**38.1 ± 5.3*****

Table 2. Female and male prepubertal organ weights (PD21-22). Body weights are for the necropsied animals only. Mean ±SD. * Indicates a statistically significant difference from controls in a Dunnett's test ($p<0.05$).

	Control	**5µg/kg**	**15µg/kg**	**50µg/kg**
Female PND22	(n=7)	(n=5)	(n=8)	(n=7)
Body weight (g)	42.3±4.5	39.0±2.2	**37.6±4.3***	**38.0±5.7***
Liver (mg)	1528±207	1379±144	1360±197	1393±205
Thyroid gland (mg)	8,0±4.01	5.2±1.55	6.7±3.59	5.8±2.57
Uterus (mg)	26,7±3.7	25.2±5.3	25.6±6.0	27.2±10.5
Ovaries (mg)	15,1±3.4	14.5±2.9	14.1±1.5	**10.4±3.6***
Male PD21	(n=8)	(n=5)	(n=9)	(n=7)
Body weight	39.4±4.3	38.2±5.0	36.6±5.2	33.7±3.9
Liver (mg)	1333.2±168.5	1367±156.3	1236,0±232.2	1132,6±240.6
Testis (mg)	196.1±21.5	194.9±17.0	183,1±24.9	172.5±24.4
Epididymides	35.8±5.2	32.0±3.5	29.6±5.4	32.0±5.6
Prostate (mg)	25.5±5.3	22.4±4.4	**18.7±5.1***	23.8±2.6
Seminal vesicle (mg)	20.4±5.2	18.8±4.2	17.0±4.5	19.5±4.1
Bulbourethral glands	2.3±0.6	2.4±0.3	2.0±0.9	1.9±0.4
LABC (mg)	35.7±5.9	33.5±4.7	29.3±5.9	26.8±6.2
Prostate (g)	0.45±0.1	0.43±0.1	0.44±0.1	0.50±0.1
Vesicula seminalis (g)	1.25±0.3	1.31±0.2	1.22±0.2	1.27±0.2
gl. Bulbourethralis (g)	0.09±0.04	0.10±0.03	0.10±0.02	0.10±0.02
M. levator ani (g)	0.95±0.2	0.83±0.1	0.96±0.1	0.92±0.1

Table 3. Postpubertal female and male organ weights. *Indicates a statistically significant different from controls ($p<0.05$) in ANOVA with body weight as a covariate.

	Control	**5µg/kg**	**15µg/kg**	**50µg/kg**
Female PD50	(n=8)	(n=8)	(n=8)	(n=8)
Body weight	143±8	141±14	140±8	136±8
Uterus (g)	0.64±0.33	0.41±0.20	0.47±0.25	0.44±0.22
Uterus drained (g)	0.30±0.12	0.24±0.04	0.32±0.09	0.29±0.07
Ovaries (mg)	62.8±7.9	58.5±13.3	66.1±9.8	61.7±10.7
Female PD90	(n=8)	(n=7)	(n=10)	(n=8)
Body weight (g)	209±8	205±3	208±21	203±1
Liver (g)	6.7±0.4	**7.3±0.65***	6.8±0.8	6.9±0.6
Uterus (g)	0.42±0.09	0.44±0.15	0.38±0.07	0.40±0.09
Uterus drained (g)	0.41±0.08	0.40±0.08	0.37±0.07	0.38±0.08
Ovaries (mg)	95.1±9.2	91.6±13.4	86.7±8.8	90.3±13.3
Male PD55	(n=8)	(n=10)	(n=10)	(n=8)
Body weight	214±18	206±15	212±20	197±8
Male PD90	(n=8)	(n=8)	(n=10)	(n=8)
Body weight	351±34	354±17	347±38	354±16
Liver (g)	11.8±1.4	11.6±0.6	11.2±1.5	11.5±0.6
Testis (g)	3.5±0.3	3.5±0.2	3.5±0.3	3.4±0.2
Epididymides (g)	0.57±0.05	0.58±0.03	0.56±0.05	0.55±0.05
Prostate (g)	0.45±0.1	0.43±0.1	0.44±0.1	0.50±0.1
Vesicula seminalis (g)	1.25±0.3	1.31±0.2	1.22±0.2	1.27±0.2
gl. Bulbourethralis (g)	0.09±0.04	0.10±0.03	0.10±0.02	0.10±0.02
M. levator ani (g)	0.95±0.2	0.83±0.1	0.96±0.1	0.92±0.1

Table 4: Number and prevalence of males with a given histological findings in ventral prostates PD90

Ventral prostate histology		Control	5µg/kg	15µg/kg	50µg/kg
Dominating appearance of acini	Regular	7/8 (88%)	3/8 (38%)	4/10 (40%)	6/8 (75%)
	Papillary	1/8 (13%)	5/8 (63%)	6/10 (60%)	2/8 (25%)
Dominating epithelia type	Cuboidal	0/8 (0%)	0/8 (0%)	0/10 (0%)	1/8 (13%)
	Columnar	5/8 (63%)	3/8 (38%)	4/10 (40%)	5/8 (63%)
	High columnar	3/8 (38%)	5/8 (63)	6/10 (60)	2/8 (25%)
Epithelial atrophy	Score 0	3/8 (38%)	3/8 (38%)	6/10 (60%)	3/8 (38%)
	Score 1	3/8 (38%)	5/8 (63%)	3/10 (30%)	4/8 (50%)
	Score 2	2/8 (25%)	0/8 (0%)	1/10 (10%)	1/8 (13%)
Epithelial hyperplasia	Score 2	6/8 (75%)	4/8 (50%)	4/10 (40%)	5/8 (63%)
	Score 3	2/8 (25%)	4/8 (50%)	6/10 (60%)	3/8 (38%)
Reactive epithelial hyperplasia		2/8 (25%)	4/8 (50%)	3/8 (30%)	2/8 (25%)
Total inflammation	Score 0	1/8 (13%)	0/8 (0%)	2/10 (20%)	1/8 (13%)
	Score 1	4/8 (50%)	4/8 (50%)	5/10 (50%)	4/8 (50%)
	Score 2	3/8 (38%)	2/8 (25%)	0/10 (0%)	2/8 (25%)
	Score 3	0/8 (0%)	2/8 (25%)	3/10 (30)	1/8 (13%)

Figure legends

Figure 1. Female external genital malformations (cleft phallus). A, control animal with a shallow cleft. B, female from high dose group with a deep cleft/ large urethral slit length. C, schematic drawing of female B showing how the urethral slit length was measured. D, Prevalence of females with deep clefts (large urethral slit length) in females PD50. E, Mean urethral slit length PD90 (in units of the scale).

Figure 2. Ano-genital distance index (AGDI). Ano-genital distance adjusted for body weight for male and female offspring PD14.

Figure 3. Number of nipples in female offspring PD14.

Figure 4: Adult female mammary glands. A, Most developed type of lobule in female whole mounts PD50. B, Histological evaluation of alveolar epithelium PD50. C, Histological evaluation of dilatation of mammary ducts PD90.

Figure 5: Adult male mammary glands. A, male mammary glands reaching the lymph node, evaluated in whole mounts PD55. B, Hypertrophy of mammary epithelium evaluated in histologic sections PD90.

fig1 female malformations.eps

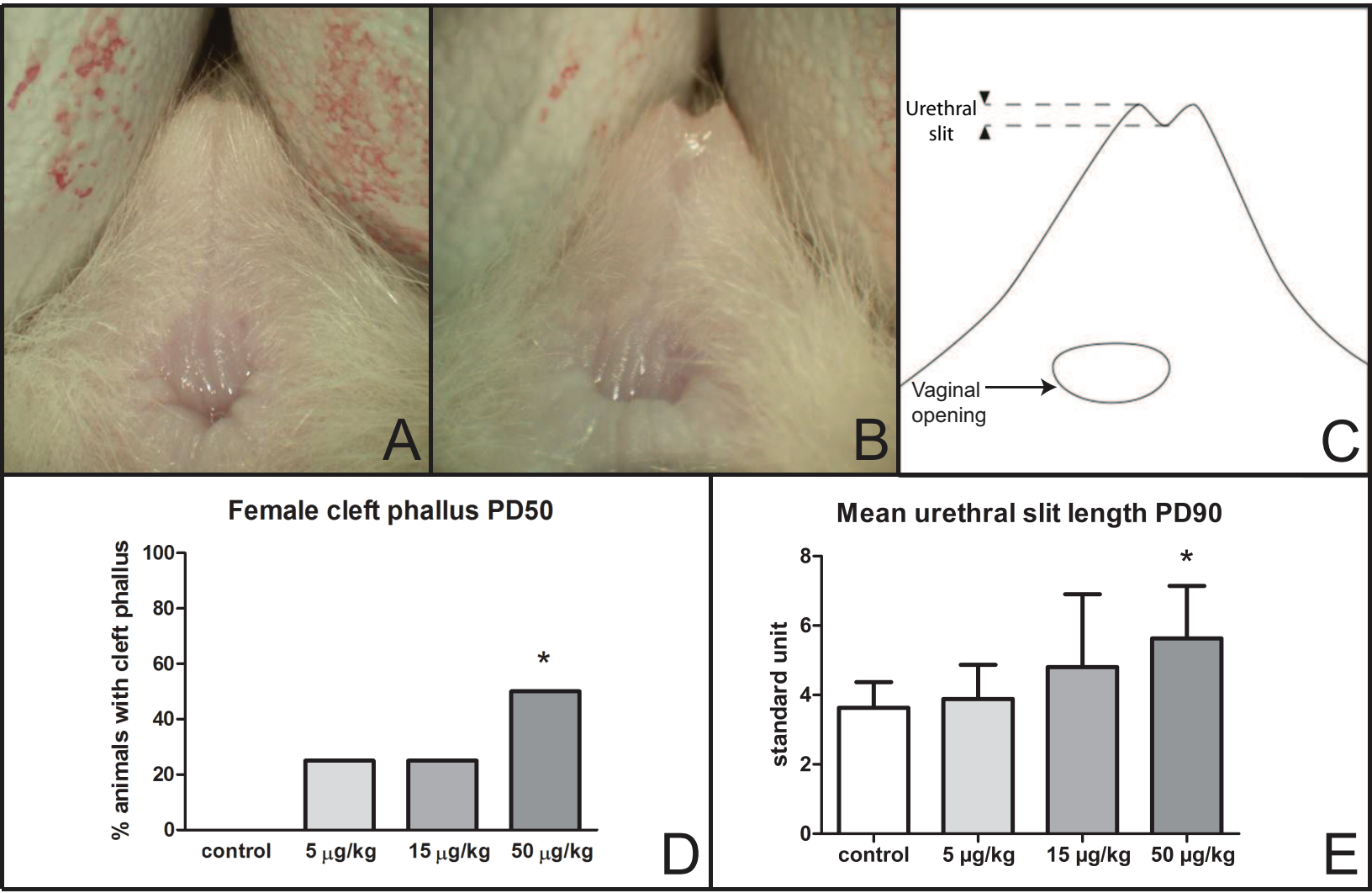

fig2 AGDI.tif
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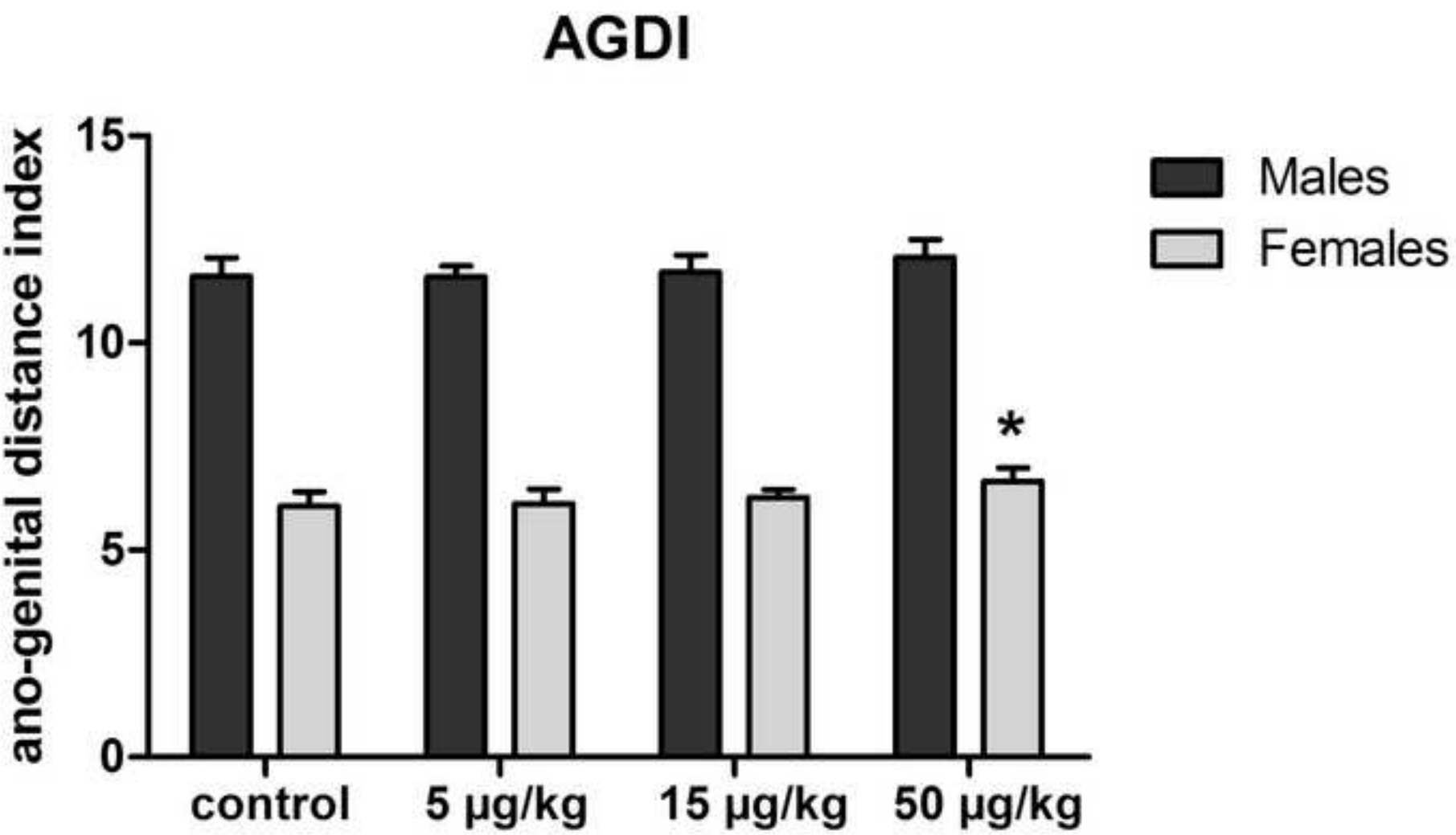

fig3 Nipples females.tif
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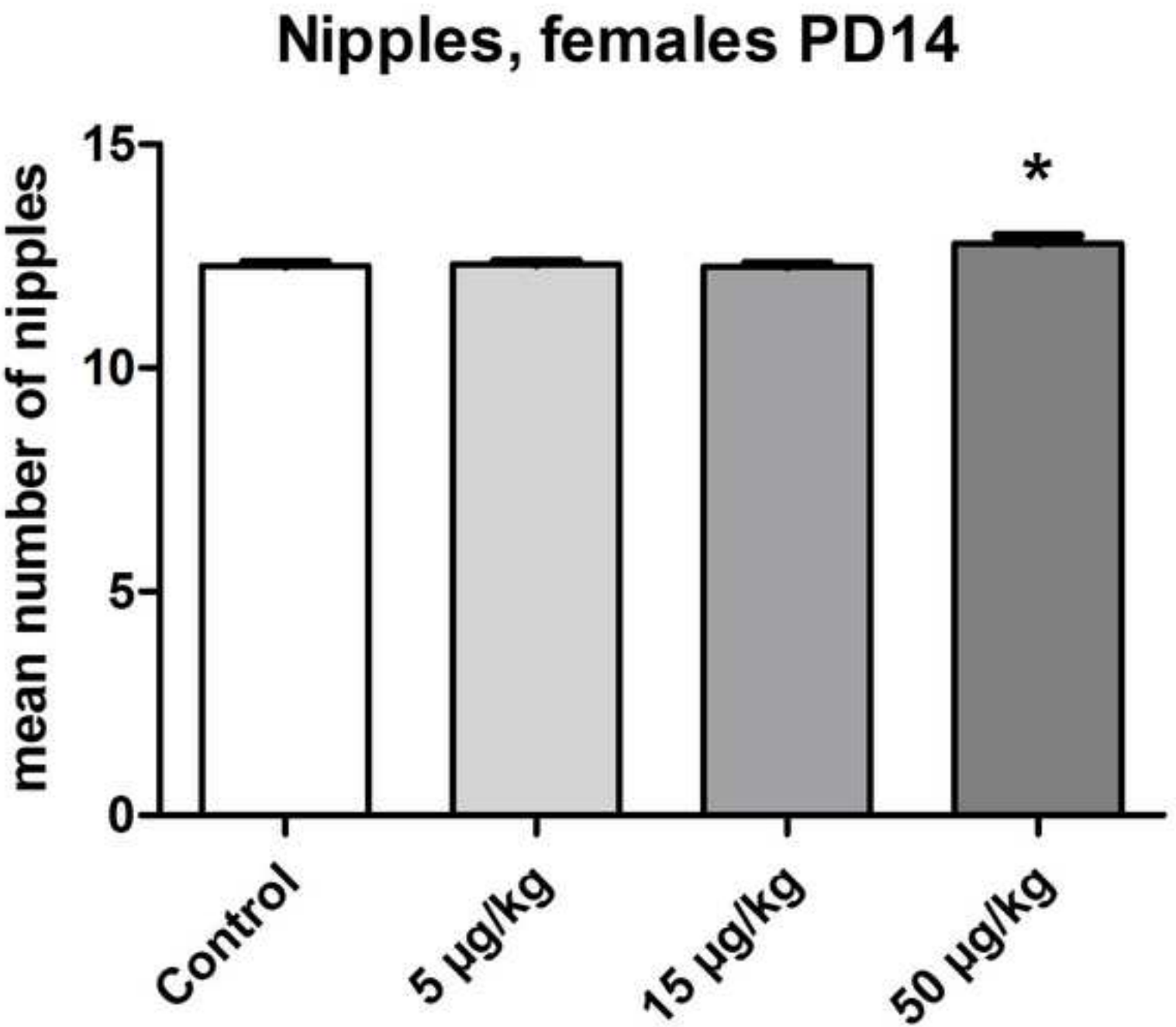


fig4 Female mammary glands.eps

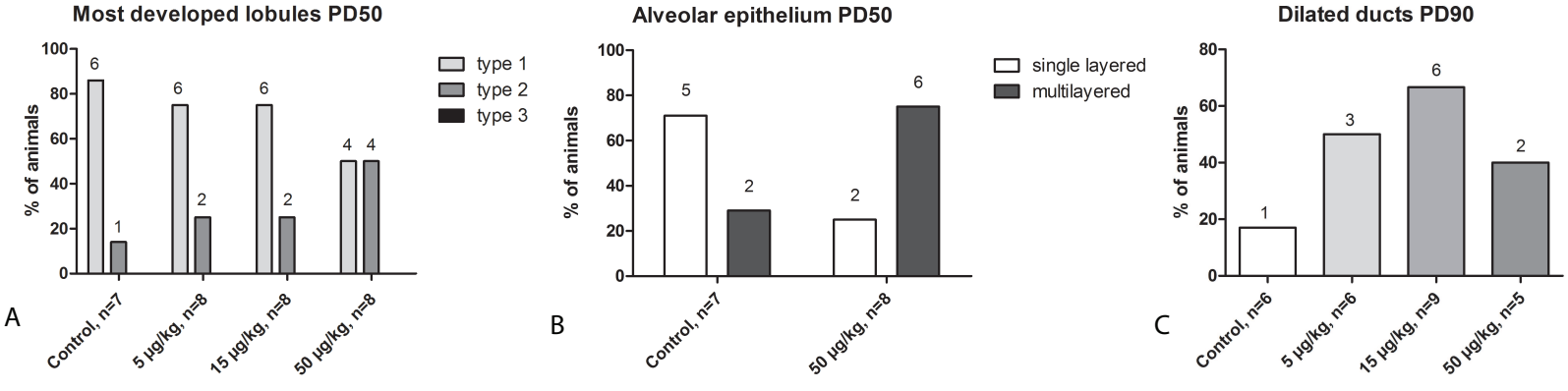
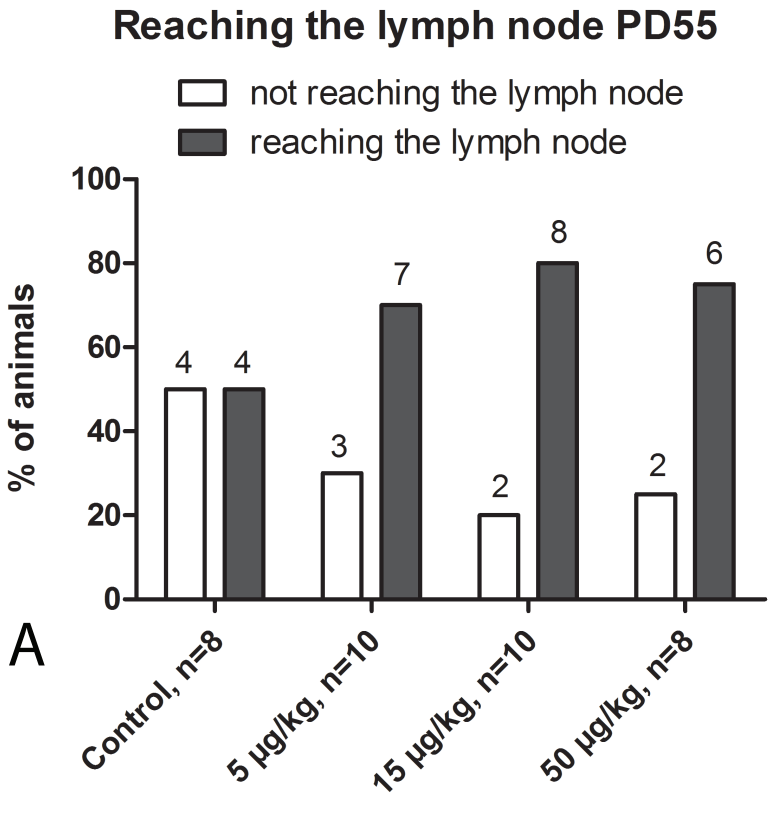


fig5 Male mammary glands.eps



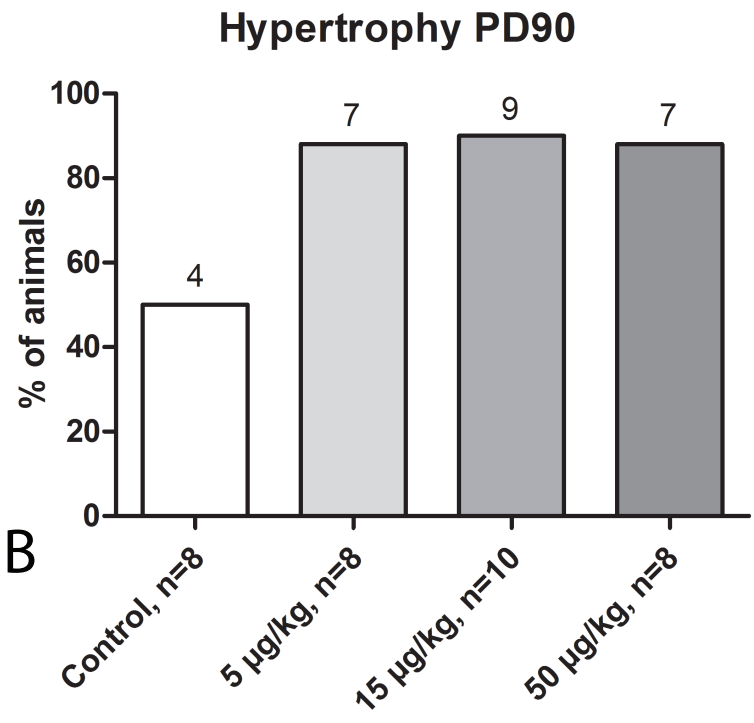


fig6 prostate gene expression.tif
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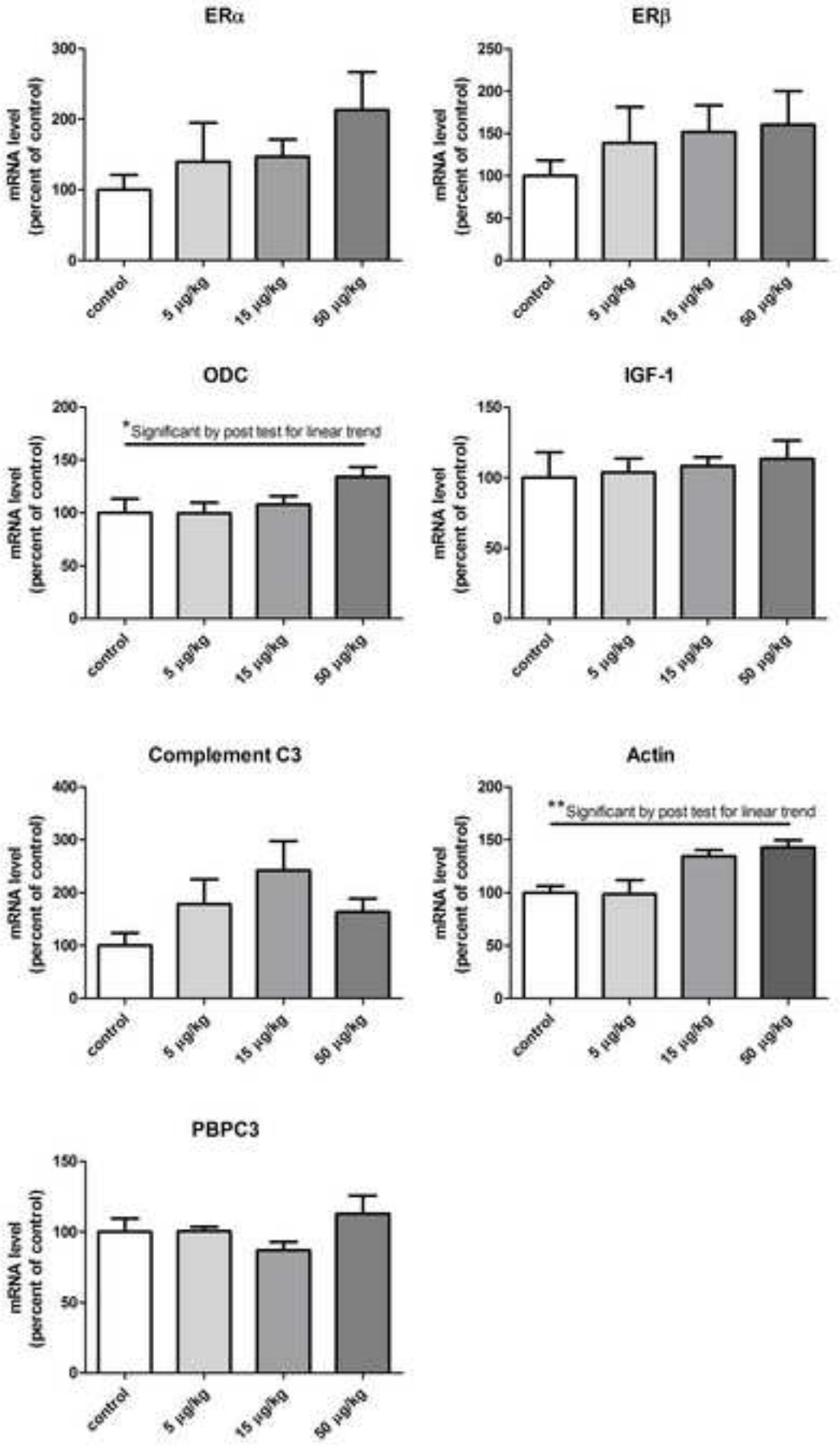

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