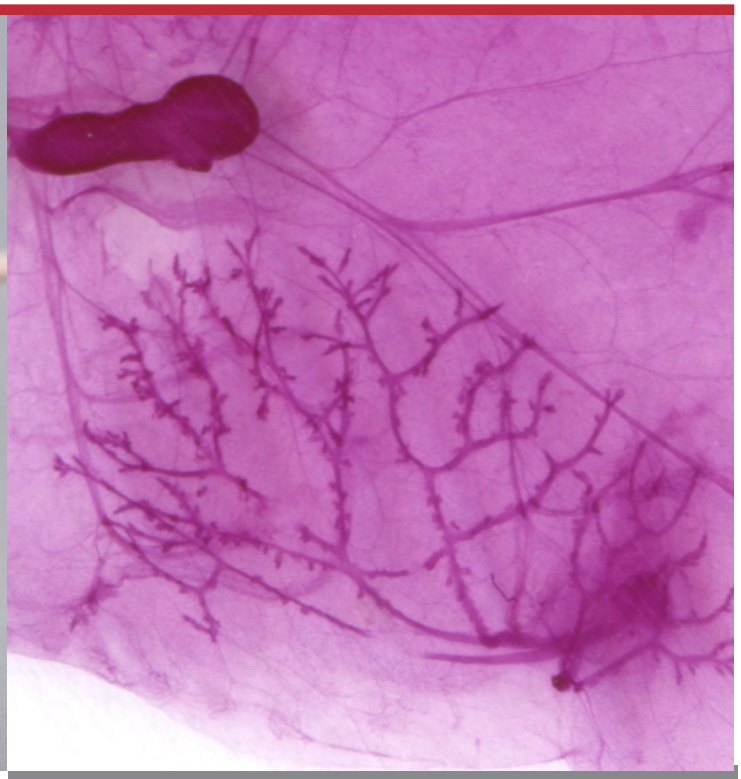
Endocrine disrupting chemicals: Effects on mammary gland development and female genital malformations



Karen Riiber Mandrup
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Data sheet

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Preface and acknowledgements

This PhD project was carried out in the division of Toxicology and Risk Assessment at the National Food Institute at the Danich Technical University (DTU) under supervision of Ulla Hass, Julie Boberg and Kirsten Pilegaard. The present project was funded by the Danish Environmental Protection Agency and the Nordic Chemicals Group.

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List of abbreviations

AGD	Ano-genital distance
Amix	Mixture of environmentally relevant chemicals suspected to have anti-androgenic properties: DBP, DEHP, Vinclozolin, Prochloraz, Procymidone, Linuron, Epoxiconazole, P,p'-DDE
ANOVA	Analysis of variance
BPA	Bisphenol A
BW	Body weight
CP	Prevalence of females with a clefted genital papilla
DBP	Di-n-butyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DL	Distance to the lymph node
ECHA	European Chemical Agency
EDCs	Endocrine disrupting chemicals
Emix	Mixture of environmentally relevant chemicals suspected to be estrogenic: 4-MBC, OMC, Bisphenol A, Butyl paraben
EW	Embryonic week
GD	Gestation day
H&E	Hematoxylin & eosine
LG	Longitudinal growth of mammary glands
Ln	Lymph node (plural: lnn)
LOEL	Low observed effect level
4-MBC	4-methyl-benzylidene camphor
MG	Mammary gland
N	Number of animals per group observed for the endpoint
NA	Not applicable
ND	Not determined
NS	Not statistically significant
NOAEL	No observed adverse effects level
NR	Nipple retention
OECD	Organisation for Economic Co-operation and Development
OMC	Octyl methoxycinnamate
PD	Pup day
PM	Paracetamol
PND	Post natal day
P,p'-DDE	Pesticide (dichlorodiphenyl-trichloroethane: DDT) metabolite
REACH	Registration, Evaluation and Authorization of Chemicals
TEB	Terminal end bud
TDLU	Terminal ductal lobular units
TG	Transverse growth of mammary glands
TotalMix	Mixture of estrogenic and anti-androgenic chemicals (Emix, Amix and paracetamol combined)
USL	Urethral slit length
WM	Whole mount

Summary

BACKGROUND: Endocrine disrupting chemicals (EDCs) may contribute to reproductive changes in boys in the Western world, however, less is known about influence of EDCs in women. The incidence of precocious breast development is increasing in USA and Europe and mammary gland development has been suggested as particularly sensitive to endocrine disruption. Mammary gland examination in toxicological studies may be useful for improving knowledge on possible influences of EDCs on human mammary glands and also be useful for detection of endocrine disrupting effects of chemicals as part of safety testing. To improve knowledge on possible influences of endocrine disrupters on female reproductive system, the effects of EDCs on genital malformations in females and the development of mammary glands were studied in the present project.

AIMS: The aims for the studies on male and female mammary gland development and female genital malformations were (i) to investigate the effects of EDCs with estrogenic or anti-androgenic mode of action, (ii) to develop methods for evaluation of the effects of EDCs in offspring exposed during foetal and postnatal development until weaning and (iii) to investigate the sensitivity of these methods by examining the effects of perinatal exposure to different environmentally relevant EDCs in offspring.

METHODS: Rat studies were used as a model for humans. Rat dams were exposed to EDCs during pregnancy and the lactation period. Female and male offspring were examined for changes in mammary gland development before puberty in whole mounted mammary glands and in adults in histological sections of the mammary glands. Moreover, female offspring were evaluated for external genital malformations. The EDCs studied for mammary gland effects were the estrogenic compounds ethinyl estradiol and genistein, a mixture of phytoestrogens, and a mixture of environmentally relevant estrogenic EDCs of various origins. Moreover, mixtures of anti-androgenic chemicals were investigated. These include a mixture of pesticides and a mixture of environmentally relevant anti-androgenic EDCs of various origins. Finally, a mixture with environmentally relevant EDCs with dissimilar modes of action was studied. Female genital malformations were investigated for the compounds ethinyl estradiol, bisphenol A and epoxiconazole as well as the mixtures of environmentally relevant EDCs.

RESULTS: Mammary glands in rats were sensitive to EDCs. EDCs with estrogenic mode of action appeared to increase mammary outgrowth in prepubertal female rats and a potent model compound, ethinyl estradiol, increased the density in females and males and the number of terminal end buds in male rats. Histological examination showed changes in epithelial morphology in male (hypertrophic epithelium) and female (lobuloalveolar morphology) mammary glands in adult rats exposed to phytoestrogens. Anti-androgenic chemicals showed signs of feminisation of adult male mammary glands. No effects of anti-androgens were observed in female mammary glands. The histological changes observed in adult female and male mammary glands were not present consistently in the groups of estrogenic or anti-androgenic chemicals and may be due to other modes of action of the chemicals. Female genital malformations were affected by the potent estrogenic chemical ethinyl estradiol, only.

In studies on exposure to anti-androgens, other endpoints, such as nipple retention showed effects in male rats at dose levels where no effects were observed in male or female mammary glands or female external genitals. However, in studies on estrogenic chemicals, marked effects on prepubertal female rat mammary glands were observed at lower levels than those affecting other endpoints studied.

CONCLUSION: The present findings in rats suggest that EDCs may affect mammary gland development in women and men, although risk assessment including comparison with exposure is necessary to draw conclusion on this. Histological examination of mammary glands are included in the extended one-generation OECD guideline studies, however, risk assessment of estrogenic chemicals may overlook the effects on mammary glands if outgrowth in females PD22 is not investigated. Further studies are necessary to confirm the high sensitivity of the distance to the lymph node in female mammary gland whole mounts and to validate this endpoint.

Resumé (summary in Danish)

BAGGRUND: Det har vist sig, at hormonforstyrrende stoffer kan bidrage til nogle af de forandringer, man i dag ser i drenges reproduktionssystem i den vestlige verden. Man ved imidlertid mindre om hormonforstyrrende stoffers betydning i kvinder. I USA og Europa er der en stigende forekomst af piger, der udvikler bryster i en tidlig alder, og studier har vist, at brystudviklingen kan være følsom for hormonforstyrrende stoffer. For at øge vores viden om de mulige effekter af hormonforstyrrende stoffer på menneskers brystudvikling, kan undersøgelse af brystudviklingen i toksikologiske studier være vigtig. Sådanne studier er også vigtige for at opdage hormonforstyrrende effekter som et led i risikovurderingen af kemikalier. For at lære mere om hormonforstyrrende stoffers påvirkning af hunners reproduktionssystem, blev effekterne af hormonforstyrrende stoffer på udviklingen af de ydre hunkønsorganer og brystvævet i både hunner og hanner undersøgt i rotter i dette projekt.

FORMÅL: Formålet med studierne på brystvævet og de ydre hunkønsorganer var:

1) at undersøge effekterne af østrogene og anti-androgene hormonforstyrrende stoffer.
2) at udvikle metoder til at vurdere effekterne af hormonforstyrrende stoffer i afkom, der har været eksponeret under fosterudviklingen og under diegivningsperioden.
3) at vurdere følsomheden af metoderne ved at undersøge effekterne af perinatal eksponering for hormonforstyrrende stoffer.

METODER: Rotter blev brugt som model for mennesket. Hunrotter blev udsat for hormonforstyrrende stoffer under drægtighed og efter fødsel, indtil ungerne blev fravænnet. Brystudviklingen i hun- og hanungerne blev undersøgt i brystvævet i sin helhed (whole mounts), i unge dyr inden de gik i pubertet (præpubertalt) og i histologiske snit af brystvævet efter kønsmodning. Hunungerne blev også undersøgt for misdannelser af de ydre kønsorganer. Effekterne på brystvævet blev undersøgt for de østrogene stoffer ethinyløstradiol og genistein, en blanding af fytoøstrogener og en blanding af miljørelevante østrogene hormonforstyrrende stoffer. Derudover blev effekterne af nogle blandinger af antiandrogener undersøgt. Disse indbefattede en blanding af pesticider og en blanding af miljørelevante antiandrogene kemikalier. Endelig blev en blanding af hormonforstyrrende stoffer med forskellige virkningsmekanismer undersøgt for effekter

i brystvævet. Effekter på de ydre hunkønsorganer blev undersøgt for ethinyløstradiol, bisphenol A, epoxiconazol og for de tre forskellige blandinger af miljørelevante hormonforstyrrende stoffer.

RESULTATER: Brystudviklingen var følsom for hormonforstyrrende stoffer. Østrogene hormonforstyrrende stoffer gav en øget udvækst af de præpubertale hunners brystvæv, og det potente modelstof, ethinyløstradiol, gav en øget densitet i hunner og hanner samt flere ”terminal end buds” i hanner. Histologisk vurdering af brystvæv fra voksne rotter viste forandringer i morfologien af epithelet i hanner (hypertrofisk epithel) og hunner (lobuloalveolær morfologi) efter fytoøstrogen eksponering. Antiandrogener så ikke ud til at påvirke hunnernes brystudvikling, hvorimod voksne hanners brystvæv viste tegn på feminisering. Der tegnede sig ikke noget mønster for de histologiske forandringer i de voksne dyr eksponeret for østrogene eller antiandrogene kemikalier perinatalt. Dette kan muligvis skyldes andre virkningsmekanismer eller forskellige dosisniveauer af de enkelte kemikalier. De ydre hunkønsorganer blev kun påvirket af det potente østrogene stof, ethinyløstradiol.
I studierne med antiandrogener blev der påvist effekter i andre endpoints, såsom ”nipple retention” (tilbageholdelse af brystvorter) i hanner, ved doser, hvor der ikke blev påvist forandringer i brystvæv fra hunner eller hanner eller i de ydre hunkønsorganer. Til gengæld blev der i studierne med østrogene stoffer påvist effekter i hunrotters brystvæv ved doser, hvor ingen effekter blev påvist i andre endpoints.

KONKLUSION: Disse fund i rottestudierne peger på, at hormonforstyrrende stoffer kan påvirke brystvævets udvikling i mænd og kvinder. Risikovurdering med sammenligning af eksponeringsniveau er dog nødvendig for at kunne drage konklusioner på dette område. Histologisk vurdering af brystvæv er med i ”extended one-generation” OECD guideline studierne. Men effekter på brystvævet i præpubertale rotter vil blive overset, hvis der foretages risikovurdering af østrogene stoffer på basis af et sådant studie, hvor kun de voksne dyrs brystvæv bliver undersøgt. Flere studier er dog nødvendige for at bekræfte følsomheden af afstanden til lymfeknuden i whole mounts af præpubertale hunner og for at kunne validere dette endpoint.

List of papers included

The following papers are included in the PhD thesis. Endpoints relating to mammary gland morphology evaluated in whole mounts and histological slides and evaluation of female external genital malformations are part of the PhD.

Paper 1: Karen Riiber Mandrup, Ulla Hass, Sofie Christiansen, Julie Boberg. Perinatal ethinyl estradiol alters mammary gland development in male and female Wistar rats. International Journal of Andrology 2012, 35: 385-396.

Paper 2: Karen Riiber Mandrup, Pernille Rosenskjold Jacobsen, Louise Krag Isling, Marta Axelstad, Karin Dreisig, Niels Hadrup, Anne Marie Vinggaard, Ulla Hass, Julie Boberg. Effects of perinatal ethinyl estradiol exposure in male and female Wistar rats. (Submitted to Reproductive Toxicology, 2013)

Paper 3: Julie Boberg, **Karen Riiber Mandrup**, Pernille Rosenskjold Jacobsen, Louise Krag Isling, Niels Hadrup, Line Berthelsen, Anders Elleby, Maria Kiersgaard, Anne Marie Vinggaard, Ulla Hass, Christina Nellemann. Endocrine disrupting effects in rats perinatally exposed to a dietary relevant mixture of phytoestrogens (Submitted to Reproductive Toxicology, 2013)

Paper 4: Pernille Rosenskjold Jacobsen, Marta Axelstad, Julie Boberg, Louise Krag Isling, Sofie Christiansen, **Karen Riiber Mandrup**, Line Olrik Berthelsen, Anne Marie Vinggaard, Ulla Hass. Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. Reproductive Toxicology 2012, 34(2): 237-250.

Paper 5: Karen Riiber Mandrup, Julie Boberg, Anne Stilling Pedersen, Mette Sidsel Mortensen, Jennifer Jørgensen, Katrine Højholt Kristensen, Ulla Hass. Mammary gland effects of perinatal exposure to environmentally relevant endocrine disrupting chemicals. (Draft manuscript/ in preparation).

Appendix 1: Historical control data in whole mounts – differences in strains and sub-strains

Appendix 2: Study report presenting evaluation of female external genital malformations

Contents

1. Introduction

Endocrine disrupting chemicals (EDCs) are chemicals that agonise or antagonise the effects of hormones in the mammalian body. EDCs affecting the sexual hormones may be classified as estrogenic or anti-androgenic, although a compound may have multiple modes of action. Increased prevalences of poor semen quality, hypospadias and testis cancer in Danish men have been reported in the last two decades as reviewed by Skakkebæk et al. (2006). Such adverse changes may decrease fertility in men and increased exposure to EDCs may contribute to the changes observed [Skakkebaek et al. 2006]. However, EDCs may also affect women. During the last two decades a decrease in the age of onset of puberty and breast development in girls has been reported in USA and Europe [Sorensen et al. 2012]. Precocious puberty can lead to psychosocial problems for the young girls and increase the risk for developing breast cancer later in life [Golub et al. 2008; Medina 2005]. Studies in rodents have shown a link between estrogenic EDCs and enhanced mammary development [Cotroneo et al. 2002; Murrill et al. 1996], suggesting that estrogenic EDCs play an important role in precocious breast development in girls [Aksglaede et al. 2009; Wohlfahrt-Veje et al. 2012]. *In vitro* and *in vivo* studies on chemicals humans are exposed to in our daily life have been shown to be endocrine disrupting, and this may contribute to the high prevalence of breast cancer in women.

In the European Union, chemicals present on the market in large quantities are regulated by authorities. Manufacturers and importers must demonstrate human safety of the chemicals by supplying experimental data on the chemicals. The OECD guidelines describe approved methods for toxicological and reproductive toxicity studies *in vitro* and *in vivo*. In the *in vivo* guidelines, several endpoints sensitive to EDCs are described, and these have been found to be sensitive to anti-androgens. However, less is known about estrogen-sensitive endpoints, although estrogenic compounds are suspected of interfering with both male and female reproductive development. Endpoints evaluated in females are either difficult to assess due to changes in reproductive organs throughout the estrous cycle or due to data being evaluated as scoring data (e.g., sexual maturation and estrous cyclicity) with low statistical power. Therefore, estrogen-sensitive endpoints and endpoints easy to assess in female rodent studies are desirable in the toxicological studies for the risk assessment of chemicals.

This project focuses on the investigation of endpoints sensitive to estrogenic chemicals as well as development of endpoints in female rats. Few studies have investigated genital malformations in female rodents and have shown an increased incidence after perinatal exposure to EDCs [Vilela et al. 2007; Wolf et al. 1999b]. In the present PhD project, a rat model was applied to evaluate the effects of perinatal exposure to estrogenic and anti-androgenic chemicals on mammary gland development in both sexes and the effects on female genital malformations. Mammary glands were examined for changes in morphologic development and the female external genital malformations were evaluated for measurable changes. The endpoints were evaluated as a model to investigate the effects of EDCs on human mammary development and to determine the usefulness of the endpoints in studying EDCs in reproductive toxicological studies.

The aims of this PhD project were:

- To investigate the effects of EDCs with estrogenic or anti-androgenic mode of action.
- To develop methods for evaluation of the effects of EDCs in offspring exposed during foetal and postnatal development until weaning.
- To investigate the sensitivity of these methods by investigating the effects of perinatal exposure to EDCs in offspring.

The aims are applied to the following two endpoints: mammary glands in females and males and female external genital malformations. Moreover, the endpoints are evaluated in relation to human risk assessment of EDCs.

The following hypotheses were formulated for each endpoint:

- Exposure to EDCs during foetal and prepubertal development affects mammary gland development in rats
- Female external genital development in rats is altered by EDCs
- Changes in female or male mammary glands or female genital malformations may be good predictors for endocrine disrupting effects of a chemical.

2. Background

2.1. Endocrine disrupting chemicals (EDCs)

Endocrine disrupters are defined by the World Health Organization (WHO) as “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” [WHO 2002]. Endocrine disrupters can act via a variety of mechanisms, either directly receptor mediated mechanisms (agonistic or antagonistic) or indirectly by affecting synthesis, transport or metabolism of the endogenous hormones [WHO 2002].

In sexually mature females, estrogen is an important sex hormone playing an essential role in the estrous cycle and the physiological changes related to the estrous cycle. Androgens are male sex hormones important for the male sex characteristics. The androgen testosterone plays a key role in spermatogenesis and in the foetal development of male sexual organs and the organisation of the male brain.

Several endocrine disrupting chemicals (EDCs) affecting sexual hormones may have an estrogenic or anti-androgenic mode of action. Estrogenic EDCs are compounds mimicking estrogens, e.g., by activating estrogen receptors or increasing the level of estrogen, whereas anti-androgenic compounds antagonize androgens, e.g., by blocking the androgen receptor or the level of androgens. Other modes of action may also influence reproductive development.

Several compounds have been shown to have endocrine disrupting properties. Soy products containing the estrogenic compounds such as genistein, plastic toys and food containers containing the anti-androgenic phthalates, and the fungicides epoxiconazole and procymidone are examples of such EDCs in our daily environment [Boberg et al. 2011; Ostby et al. 1999; Taxvig et al. 2007]. Although these compounds are classified as “estrogenic” or “anti-androgenic” here in the literature, it is important to note that these and most other compounds often exhibit several different modes of actions simultaneously.

2.2. EDCs and reproductive development

During foetal development, sexual hormones are crucial for the sexual differentiation, although hormones are only present at low levels [Apter 1997; Forest 1979]. Chemicals with estrogenic or anti-androgenic mode of action can perturb the hormonal balance during critical periods in development and may have implications later in life in the offspring even at low levels of EDCs. An increased incidence of men with undescended testes, low semen quality and testis cancer has been reported in Denmark and an increased exposure to EDCs in the environment may be related to the changes observed [Skakkebaek et al. 2006]. Indeed, EDCs have been shown to affect the development of genital organs in male rats. Changes such as increased incidence of hypospadias and cryptorchidism and decreased sperm count have been reported in rats exposed perinatally to EDCs [Axelstad et al. 2011; Christiansen et al. 2008; Kim et al. 2010]. The majority of effects of EDCs have been observed in male rodent studies on anti-androgens and little is known about the effects in females and effects of compounds with estrogenic mode of action.

Reproductive changes have also been observed in females. An increase in the prevalence of precocious puberty in girls, defined as breast development or appearance of pubic hair before the age of 8 years, has been reported in the USA and Europe during the last 2 decades, as reviewed by Sørensen et al. [Sorensen et al. 2012]. The mean age of onset of breast development in girls has dropped 1 year during a 15-years period in Denmark, reaching a mean age of 9.86 in 2006 [Aksglaede et al. 2009]. Precocious puberty may give rise to psycho-social and social consequences, e.g., a poor self-image, increased risk for eating disorders or drug abuse, as reviewed by Golub et al. (2008) and Patton and Viner (2007) [Golub et al. 2008; Patton and Viner 2007]. Early puberty onset may lead to a longer reproductive period, a period characterized by high estrogen levels and this has been associated with an increased risk for developing breast cancer later in life [Henderson et al. 1991; Medina 2005; Pike et al. 1981; Rudel et al. 2011]. Breast cancer is the most common type of cancer in women in Denmark (4944 women, corresponding to 22.8% of all women with cancer) (Cancerregisteret 2012, Sundhedsstyrelsen), a disease also seen in men (increased with 0.4% from 2006 to 2010 in Scandinavia) (NORDCAN) [Hodgson et al. 2004]. Breast development is observed in some men (gynecomastia) and breast cancer in men is usually more fatal than in women, as breast cancer in men is often detected at a much later stage.

The mechanisms responsible for precocious breast development are yet unknown. Hormonal measurements indicate that early breast development is not associated with early activation of the

pituitary-gonadal axis (leading to high gonadotropin or estradiol concentrations) neither can body mass index explain the early breast development [Aksglaede et al. 2009; Wohlfahrt-Veje et al. 2012]. Precocious puberty, early breast development and high incidence in breast cancer in women may be explained by increased exposure to EDCs in the environment. In a study investigating the breast development in girls from mothers working in greenhouses during early pregnancy, prenatal exposure to pesticides could be associated with breast development 1-1.5 years earlier compared to non-exposed girls [Wohlfahrt-Veje et al. 2012]. Several pesticides such as tebuconazole, epoxiconazole and prochloraz have been shown to be endocrine disrupting [Taxvig et al. 2007; Vinggaard et al. 2002] and the early breast development observed may be explained by the endocrine disrupting activity of the pesticides. In fact, various EDCs have been shown to affect mammary gland development in rodent studies and especially estrogenic compounds, such as phytoestrogens (estrogenic compounds from plants), have been reported to increase growth of mammary glands in females [Cotroneo et al. 2002; Delclos et al. 2001; Murrill et al. 1996]. Estrogenic EDCs have also been shown to induce neoplastic transformation *in vitro* and exposure to estrogenic compounds during early development increased the susceptibility to carcinogen-induced mammary tumors in rats [Fernandez and Russo 2010; Khan et al. 2007; Tan et al. 2004; Ward et al. 2000]. Thus estrogenic EDCs may affect mammary gland development and contribute to the precocious breast development seen in girls in the Western world and may contribute to the high prevalence of breast cancer in women. However, disagreement on the roles of estrogenic compounds in breast cancer has risen, as some studies have shown protective effects of estrogens against mammary cancer [Chen et al. 2003; Tou and Thompson 1999]. Focus on male breast cancer is lacking in *in vivo* studies, although a few studies have suggested that male mammary glands may be more sensitive to EDCs than female mammary glands [Latendresse et al. 2009; You et al. 2002], and other studies have suggested that some idiopathic gynecomastia may be related to EDC exposure [Henley et al. 2007].

2.3. Mammary gland development

Mammary glands are a common feature for mammals, however, the number, shape and size vary between species. Despite these differences, the embryologic development of the mammary glands is comparable between species [Cowie 1974; Hovey et al. 2002].

In rats, mammary gland development starts at gestation day (GD) 11, where the milk line is formed ventrolaterally from the cervical to the inguinal region. Between GD12 and GD13, mammary buds are formed in the milk line. When testosterone levels start rising in male fetuses between GD 13 and 15 (peak at GD19) mammary glands are sensitive to testosterone, and differentiation to either female or male mammary glands occurs [Borellini and Oka 1989; Kratochwil 1977]. At GD15, cells proliferate and form the mammary cord from which branching occurs before birth [Borellini and Oka 1989]. After birth, the development of the mammary glands continues. From the age of 14 days, 12 nipples are visible in female offspring (Figure 1), whereas male rats are born with a rudiment of the mammary gland and nipples are not present. In humans, the mammary glands follow similar developmental pattern but the milk line regresses in the caudal region, and mammary buds are formed in the thoracic region only. The embryonic mammary development in humans starts in the first trimester on embryonic week 10 and ends before birth on embryonic week 32 [Fenton 2006] (Figure 2).

After birth, the rat mammary gland undergoes two phases of development. Until weaning (22-23 days old) the growth of the glands follows the growth of the body weight (isometric growth). After weaning and until puberty, the mammary gland grows approximately three-fold faster than the body weight (allometric growth) (reviewed by Borellini and Oka 1989). Until sexual maturation, mammary glands of males and females are morphologically comparable but not identical [Cardy 1991; Ceriani 1970; You et al. 2002]. After sexual maturation rat mammary glands develop differently according to gender. Female mammary glands grow extensively (allometric growth) [Borellini and Oka 1989] passing the lymph node and filling out the mammary fat pad. In histological slides virgin female rat mammary glands are recognizable by a tubuloalveolar pattern; this typical female morphology is characterized by ducts and alveoli with distinct lumina and lined by a single layer of cuboidal epithelium [Cardy 1991; Wang et al. 2006]. Male rat mammary glands are smaller than female mammary glands and characterized by a lobuloalveolar pattern described as large lobular groups of cells arranged into alveoli with indistinct lumina and aboundant, eosinofilic and vacuoled epithelium [Cardy 1991; Wang et al. 2006]. The hormonal balance in sexually mature females varies according to the estrous cycle, which seems to have implications on the mammary glands. Lobules are further developed with each estrous cycle and full differentiation of female mammary glands is only reached at pregnancy [Russo and Russo 1978; Russo et al. 1990]. An increased proliferative index has been shown in females in metestrous, leading to a lobular morphology (type 2 with 11-25 acini or type 3 with more than 25 acini) of the adult mammary

glands in metestrous and early diestrous compared to small lobules with 10 or fewer acini (type 1) in late diestrous and proestrous [Hvid et al. 2010; Schedin et al. 2000], although Schedin et al. (2000) describes a large variation in the morphological appearance within each stage of estrous cycle [Schedin et al. 2000].

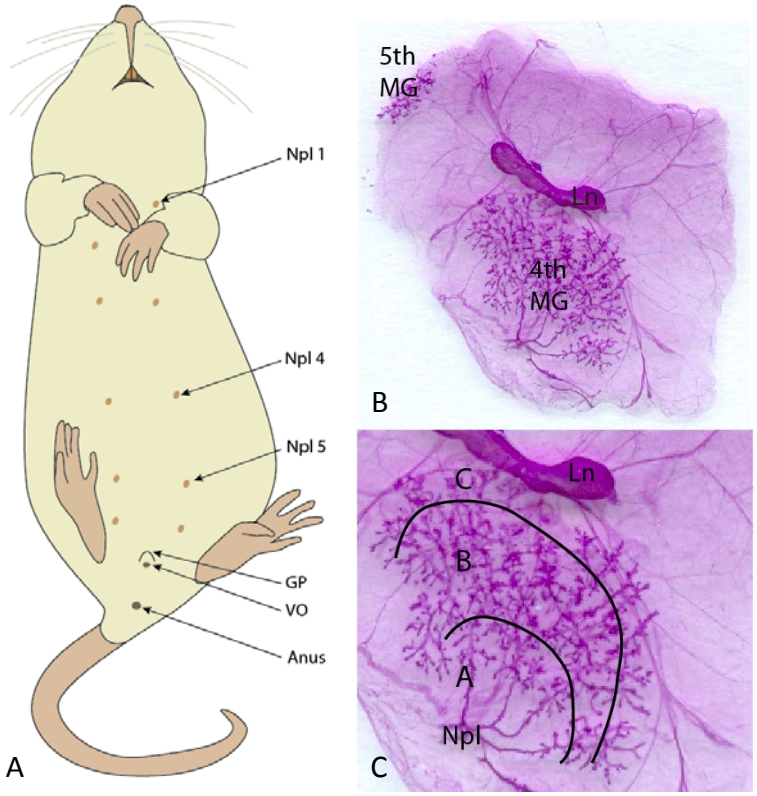

Figure 1. Female rat mammary glands. (A) Ventral view of a sexually mature female rat. Twelve nipples (Npl) are present, aligned by pairs from the cervical region (nipple pair nr. 1) to the inguinal region (nipples 5 and 6). The genital papilla (GP) is situated anterior to the vaginal opening (VO). (B) Mammary gland (MG) whole mount from 22 days old female rats scanned on a flatbed scanner (4200 dpi). (C) Zones A, B and C defining areas of most to least differentiated parts of the mammary glands. Ln= lymph nodes. (Figures by Karen Riiber Mandrup).

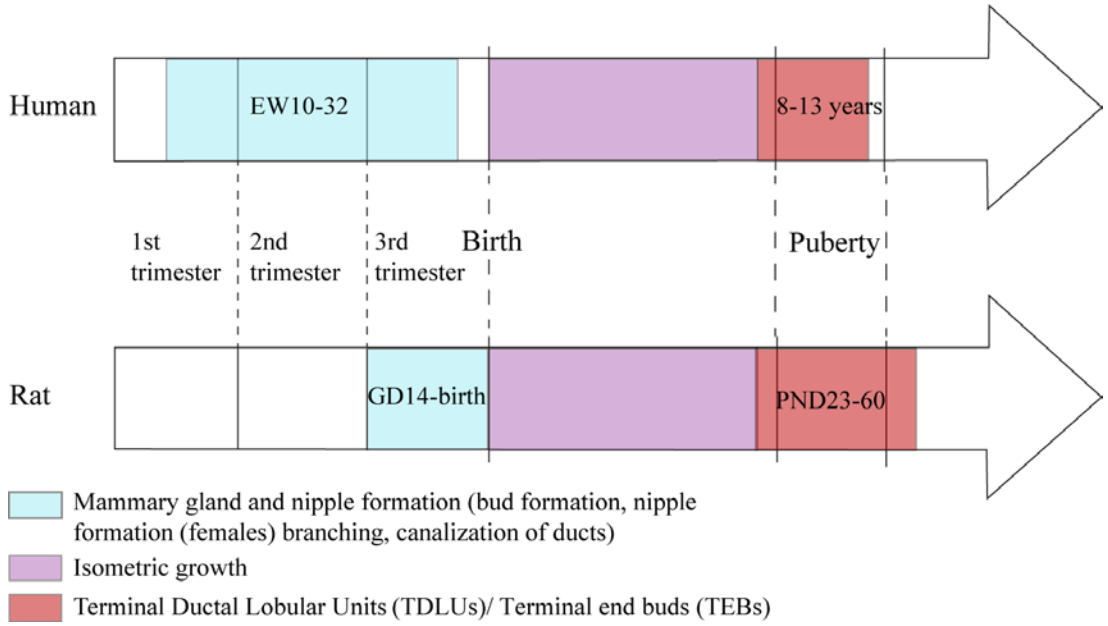

Figure 2. Timing of mammary development in rats and humans. EW: embryonic week. PND: post natal day. GD: gestation day. (Figure by Karen Mandrup)

A number of differences between rat and human mammary gland development are present. In rats, ducts grow into the fat pad whereas human ducts grow along connective tissue septa and the lobular structures do not grow into the fat tissue [Russo et al. 1990]. Moreover, rat mammary gland can be divided into zones from a well differentiated area close to the nipple (zone A) and the least differentiated zone furthest away from the nipple (zone C) (Figure 1); such systematic gradient in development is not found in humans where the growth is more labyrinthic and varying degree of differentiation is found in the breast [Russo et al. 1990]. Additionally, a good correlation between age and gland development can be found in rats, of which none is found in humans where different degrees of development is observed in breasts of same aged women [Russo et al. 1990].

Hormones play a crucial role in mammary gland development during fetal development in males and during puberty and adulthood in females [Neville et al. 2002]. In male mice and rats mammary gland development is controlled by androgens during fetal development, blocking the formation of nipples [Goldman et al. 1976; Kratochwil 1977; Raynaud and Frilley 1947]. After birth several hormones are involved in the development of the female mammary gland, however the mechanisms are not fully understood. In general, studies in rodents have found estrogen, progesterone and androgens to be important for mammary gland development. Estrogen is responsible for ductal elongation and branching, but seems to act in combination with growth hormone or prolactin

[Borellini and Oka 1989; Sourla et al. 1998]. Progesterone causes side branching and alveolar formation [Brisken 2002; Sourla et al. 1998]. Androgens are responsible for the male-like development of the mammary glands. In adult males androgens give rise to lobuloalveolar growth with a profuse growth of lobular units present around 2 months of age and multilayered epithelium in ducts and alveoli from the age of 3 months [Ahren and Etienne 1957; Sourla et al. 1998]. In humans, estrogen induces cell proliferation leading to ductal growth and progesterone enhances differentiation of the gland by developing acini [Borellini and Oka 1989].

Despite differences between human and rat mammary glands, the development of the glands has many similarities. The rat mammary gland appears to be a good model for human mammary gland development and useful to study the effects of chemicals on the gland development.

2.4. Methods for evaluation of mammary gland effects

Three periods in the mammary development have been identified as sensitive periods for exposure to EDCs: the prenatal phase, the prepubertal phase and pregnancy [Fenton 2006]. Exposure to chemicals during both the prenatal and prepubertal phase (*in utero* and during lactation) especially appears to affect mammary gland development [Rayner et al. 2005] and may have adverse effects later in life, e.g. an increased risk of developing mammary cancer [Knight and Sorensen 2001] (Figure 3). Rat mammary glands can be evaluated in whole mounts or in histological slides, each method with different purposes. Evaluation of mammary gland growth as a marker for early or delayed mammary development in prepubertal rats can be done in whole mounted mammary glands. For whole mounting, dissected rat mammary glands are spread and fixed as a whole on a glass slide. After processing and staining, the mammary gland is macroscopically visible in its fat pad. This is called a whole mount of a mammary gland (Figure 1). Compared to histological sections of mammary glands, the whole mammary gland can be examined in whole mounts and give an overall picture of the stage of development or morphological changes of the gland. For the evaluation of differentiation and persistent epithelial as well as morphological effects on adult mammary glands, histological slides of mammary glands can be assessed. Histological examination of the mammary glands can include investigation of the presence of receptors, proliferation, apoptosis etc. with immunohistochemical staining. Moreover, both whole mounts and histological slides can be used to investigate the effects on terminal end buds (TEBs). In rats, TEBs are undifferentiated tear-drop shaped structures in the zone C of the mammary gland (Figure 1). TEBs

are the site of extensive proliferation both responsible for the elongation and branching of ducts [Brisken 2002]. Neoplastic transformation occurs primarily in the TEBs of rats [Russo et al. 1990]. In humans, the virginal lobules called terminal ductal lobular units (TDLUs) (or lobules type 1) seem to represent an undifferentiated stage where neoplastic transformation can occur. The TEBs in rats are thus considered to be the equivalent to TDLUs in humans [Russo et al. 1990]. In rats, the number of TEBs peak before puberty at 21 days of age [Russo and Russo 1978], and an increase in the number of TEBs in rats represents an increased risk of developing mammary cancer.

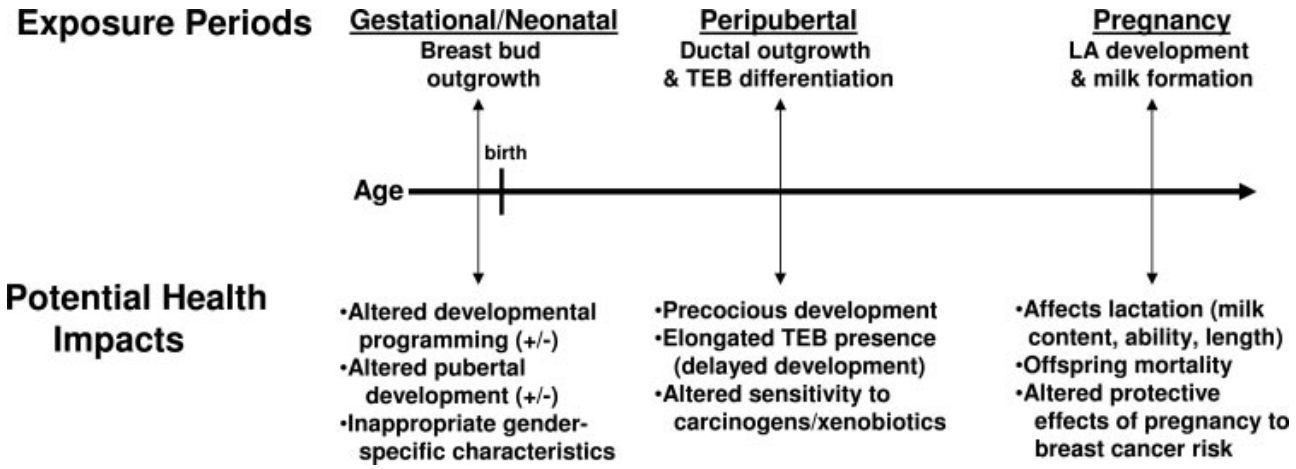

Figure 3. Timeline highlighting the critical periods of female mammary gland development most likely to be altered by EDCs and the correlating potential health effects of these exposures. Three time periods are suggested from rodent model literature. LA: lobuloalveolar. TEBs: terminal end buds. (from Fenton 2006)

Whole mounting is optimal for small mammary glands that have not yet reached the boundaries of the fat pad, e.g. prepubertal mammary glands. Growth parameters can be difficult to evaluate when mammary glands have occupied the fat pad and the limits between two neighbouring mammary glands can be challenging to distinguish.

Mammary whole mounts are most commonly evaluated for outgrowth (area, ductal elongation or the distance to the 5^{th} gland) (Figure 4), gland differentiation (number of terminal end buds, alveolar development/ types of lobules present) and the density (branching and budding) of the mammary glands [Hovey et al. 2005; Moon et al. 2007; Thomsen et al. 2006; You et al. 2002].

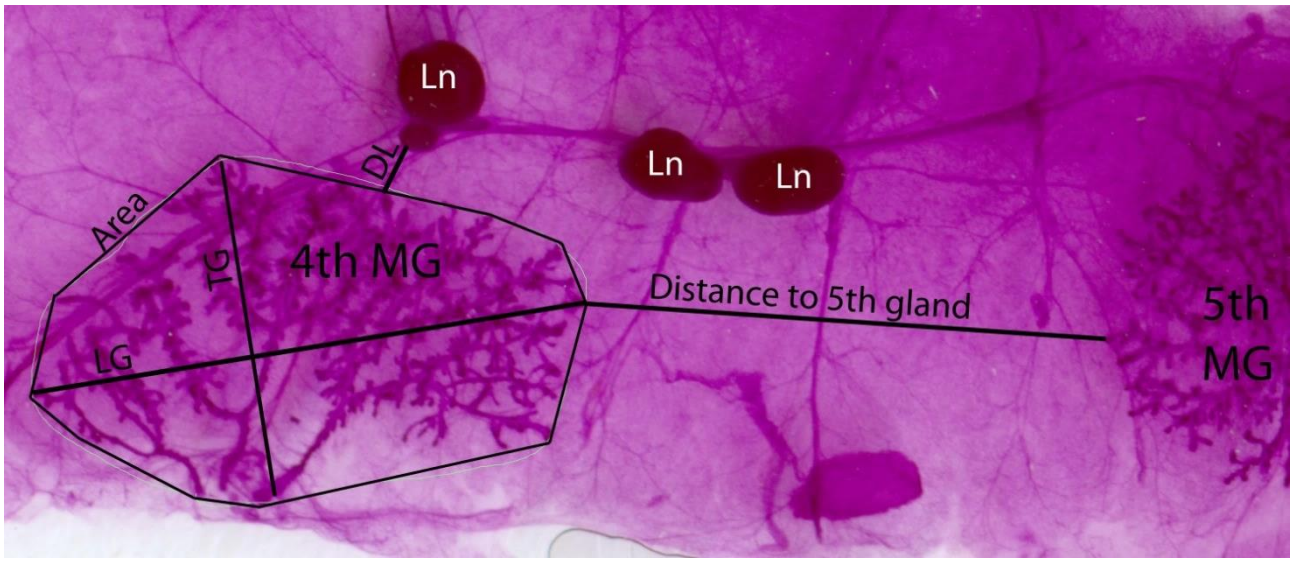

Figure 4. Example of parameters for evaluation of mammary glands in whole mounts. Measurements for ductal outgrowth are depicted. MG: mammary gland. LG: longitudinal growth. TG: transverse growth. DL: distance to the lymph nodes. Ln: lymph node.

Adult mammary glands are more easily evaluated in histological sections, where other morphological changes - cellular and epithelial changes - can be evaluated in hematoxylin and eosin (H&E) stained slides. Sectioning of the mammary gland should be done in a horizontal section, i.e. parallel to the skin [Hvid et al. 2011].

2.5. Abnormal mammary development following EDC exposure

Estrogenic chemicals such as genistein, estradiol benzoate and secoisolariciresinol diglucoside have been shown to enhance mammary development and differentiation in rats. In whole mounts, evaluation of mammary glands have revealed an increased outgrowth of mammary glands (e.g. increased area) [Murrill et al. 1996] and an increased number of TEBs [Cotroneo et al. 2002; Tan et al. 2004] in prepubertal female rats and an enhanced differentiation (increased prevalence of lobules type 2) [Murrill et al. 1996; Ward et al. 2000] in adult female rats after perinatal exposure. Bisphenol A has also been shown to increase the number of TEBs [Moral et al. 2008]. However, these findings are not consistent among studies [Fritz et al. 1998; You et al. 2002]. In male rats comparable changes have been reported. Estrogenic chemicals have been shown to increase branching in prepubertal male rats and increase the area and density of the mammary glands in adult male rats [Wang et al. 2006; You et al. 2002].

Studies evaluating the effects of EDCs on adult mammary histology have reported effects in both male and female mammary glands. Estrogenic chemicals have been shown to lead to lobular hyperplasia [Takagi et al. 2004] and increased secretion in virgin females observed as dilated secretion-filled ducts [Biegel et al. 1998]. Moreover, a shift in the dominating lobuloalveolar pattern in males to larger areas with tubuloalveolar pattern [Biegel et al. 1998] and hypertrophy of mammary epithelium, have been reported [Delclos et al. 2001]. Other EDCs like genistein, with both estrogenic and anti-androgenic modes of action, have shown to affect female mammary glands to a shift from the tubuloalveolar pattern to a more lobuloalveolar-like pattern [Delclos et al. 2001].
Few studies have investigated the effects of anti-androgens on mammary glands. Changes have been observed in pubertal or older rats. Saad et al. (2011) showed increased branching and ductal hyperplasia (multi-layered epithelium) in pubertal female rat mammary glands after perinatal exposure to vinclozolin [Saad et al. 2011]. Moreover, vacuolar degeneration and alveolar atrophy was reported in adult males exposed to di-*n*-butyl phthalate [Lee et al. 2004]. Thus, female and male mammary glands may be affected by EDCs with estrogenic or anti-androgenic modes of action with a wide range of possible changes observable in whole mounts and histological slides.

2.6. Female external genital malformations

The proper development of the female external genitals is important for female reproduction at mating and birth but also in the everyday life at urination and as a barrier for the external world. Hence, it is important to make sure that the development of the external genitals is not impaired. The development of the external genitals in foetal life of females or males is controlled by the lack or presence, respectively, of the hormone testosterone [O'Connor and Chapin 2003]. The female genitals are said to be the “default” form of the external genitals. The mechanisms controlling the development of the female external genitals are unknown, yet, it is probable that these mechanisms can be disturbed by the exposure to EDCs and this should be investigated.

In female rats, the external genitals are represented by a genital papilla, anterior to the vaginal opening (Figure 1). At the tip of the papilla where the urethra ends, is a small cleft, the urethral slit. A deep urethral slit may be referred to as a clefted genital papilla (Figure 5). In the literature, the female external genitals have been evaluated in different ways: by registering the absence or presence of a vaginal thread, by measuring the depth of the urethral slit (urethral slit length), by

determining a clefted papilla as deeper than control urethral slits or by measuring the distance between the cleft and the vaginal opening (urethral-vaginal distance).

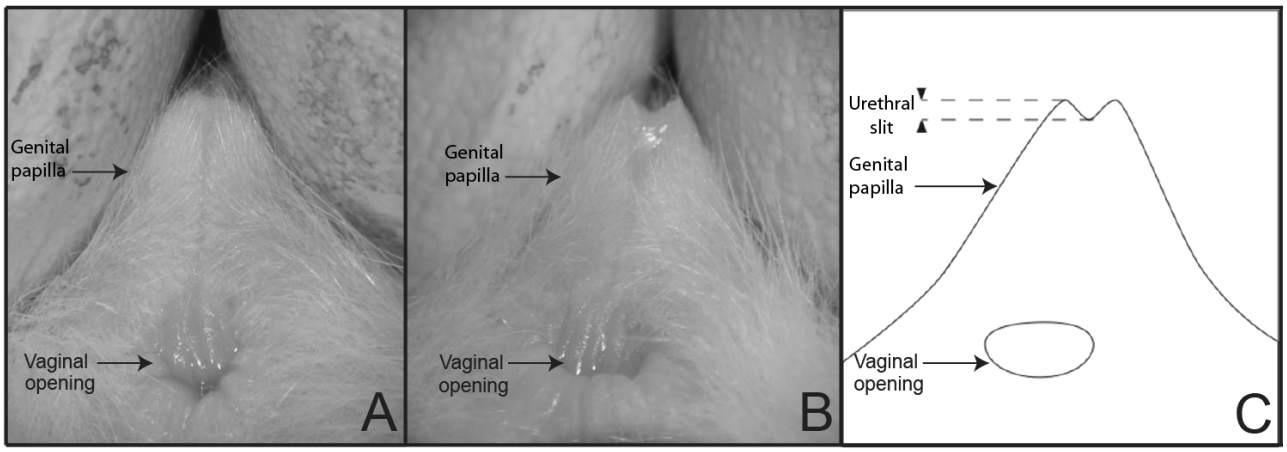

Figure 5. Malformations of female external genitals. A, A normal genital papilla from a control animal. B, A clefted genital papilla with a deep urethral slit. Female exposed to ethinyl estradiol. C, Sketch of picture B illustrating the measurement of the urethral slit length. (Figures by Karen Riiber Mandrup)

Deep urethral slits or clefted genital papillas have been demonstrated in females exposed to EDCs or endocrine disrupting model compounds with different modes of action (Table 1). Presence of a clefted papilla or increase in the urethral slit length has been described in adult rats after prenatal exposure to 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD), tamoxifen and testosterone propionate [Flaws et al. 1997; Gray et al. 1997; Gray and Ostby 1995; Wolf et al. 2002; Yamasaki et al. 2005]. Perinatal exposure to ethinyl estradiol showed an increase in the urethral slit length [Sawaki et al. 2003a; Sawaki et al. 2003b]. Wolf et al. (1999) also showed a decrease in the urethral-vaginal distance in hamsters exposed to TCDD [Wolf et al. 1999a]. These findings are evidences of external genital malformations in females after exposure to EDCs. Thus, it is of interest to investigate the female external genitals for malformations and determine if (i) the development of female external genitals is sensitive to EDCs, if (ii) there is a specific sensitive method for evaluation of the female genital malformations sensitive to EDCs and, thus, if (iii) the development of female genital malformations could be used as a marker for endocrine disruption in foetal development.

Table 1: Literature presenting results on female genital malformations after perinatal exposure to endocrine disrupting compounds. Different means of measurement: urethral slit length (USL) and prevalence of females with a clefted genital papilla (CP).

Paper	Chemical	Pre-/postnatal dosing	Animal	Changes	Age at evaluation
Gray & Ostby 1995	TCDD	+/- GD8-15	Long Evans rat	USL↑ at 1 µg/kg	Adult
Gray *et al.* 1997	TCDD	+/- GD15	Long Evans rat	CP↑ at 0.8 µg/kg USL↑ at 0.2 µg/kg	Adult
Flaws *et al.* 1997	TCDD	+/- GD11,15 or 18	Sprague-Dawley rat	CP↑ at 1 µg/kg	Pubertal
Wolf, Ostby & Gray 1999	TCDD	+/- GD11.5	Hamster	Urethral-vaginal distance ↓ at 2 µg/kg	Adult
Yamasaki *et al.* 2005	Tamoxifen	+/+ GD6-PND21	Sprague-Dawley rat	CP↑ at 0.6 µg/kg	Adult
Wolf *et al.* 2002	Testosterone propionate	+/- GD14-19	Sprague-Dawley rat	CP↑ at 0.5mg, but not at 1 mg	Adult
Sawaki *et al.* 2003a	Ethinyl estradiol	+/+ GD7-PND18	Sprague-Dawley rat	USL↑ at 50 µg/kg	Adult
Sawaki *et al.* 2003b	Ethinyl estradiol	+/+ GD7-PND18	Sprague-Dawley rat	USL↑ at 50 µg/kg, CP ↑ (15/16)	Adult

TCDD: 2,3,7,8-tetrachlordibenzo-*p*-dioxin. Arrows: parameters reported to be increased or decreased.

2.7. Regulatory considerations

To secure human safety, the European Parliament has implemented legislation regulating chemicals used in the European Union, a legislation called REACH (Registration, Evaluation and Authorization of Chemicals). In Europe, all chemicals produced or imported in amounts exceeding 1 ton per year must be registered to ECHA (European Chemical Agency) [European Parliament and Council of Europe 2006]. To be approved for the European market, such chemicals must be tested and the results have to be reported to ECHA. The studies required on a compound depend on the amounts produced or imported per year. Higher amounts of the chemical on the market require more data and more complex toxicological studies. The studies include *in vitro* and *in vivo* studies, e.g. studies for mutagenicity, acute toxicity and reproductive toxicity. The OECD test guidelines

describe study methods used for the testing according to REACH. Chemicals with endocrine disrupting activities may be detected in the 2-generation study, mandatory for chemicals exceeding 1000 tons per year. In this study, some sensitive endpoints for endocrine disrupting effects on males, known to be sensitive to anti-androgens, are included [OECD 2001]. However, effects of estrogenic chemicals may be difficult to detect. The extended one-generation study includes histological examination of mammary glands and the relevance of the young mammary gland as a sensitive endpoint to estrogens is mentioned in a footnote [OECD 2010b]. This endpoint is yet to be validated, before inclusion in the extended one-generation study. This type of study is presently only included in testing requirements for pesticides and biocides, whereas effects on mammary glands are not tested for REACH chemicals.

Most toxicological studies investigate the effects of single compounds and risk assessment of chemicals is usually done also for single compounds. However, the combination of several chemicals is more representative for human exposure. Tinwell and Ashby (2004) showed effects of a mixture that consisted of estrogenic chemicals, at doses where no effects were observed for single compounds [Tinwell and Ashby 2004]. Thus, EDCs may lead to toxicological effects if combined with other chemicals with similar modes of action, although exposure of each compound is below the no observed adverse effect level (NOAEL). Although the exact mechanisms of action of a compound are seldomly known, prediction of the mixture effects of some EDCs is possible. Dose-addition appears to be useful to predict the mixture effects observed when compounds with similar modes of action are mixed [Christiansen et al. 2012; Orton et al. 2012; Tinwell and Ashby 2004]. However, mixture effects of chemicals with different modes of action (e.g. anti-androgens and estrogens) may be more difficult to predict.

3. Experimental design and methods

To answer the aims of the PhD thesis, effects of perinatal exposure to EDCs on mammary gland development evaluated in whole mounts on PD21-22 and in histological slides in adult offspring were investigated. Moreover, the effects on female external genital malformations were investigated.

Overall, the goal was to determine morphological parameters in female or male rat mammary glands sensitive to EDCs. Single compounds or mixtures of chemicals were investigated *in vivo.* Firstly, a positive control chemical, a potent estrogenic compound, ethinyl estradiol, was studied (papers 1 and 2). Secondly estrogenic compounds from food products or environmentally relevant estrogenic chemicals were studied as single compounds (genistein) or in mixtures of several compounds (phytoestrogens or various environmentally relevant estrogenic chemicals (Emix)) (papers 3 and 5). Finally, the endpoints were investigated in offspring exposed to mixtures of anti-androgens (pesticides or chemicals with various origins (Amix)), or chemicals with dissimilar modes of action (TotalMix) or to the medicament paracetamol (papers 4 and 5). The sensitivity of female external genital malformations in Wistar rats were evaluated in the study with the positive control compound, ethinyl estradiol (paper 2), and secondly in studies with other single compounds of EDCs (bisphenol A, epoxiconazole and paracetamol) or mixtures of environmentally relevant EDCs (Emix, Amix, TotalMix) (Appendix 2). The effects were analysed to better understand how estrogenic and anti-androgenic chemicals may affect mammary glands or the female genital malformations in combination with other chemicals with similar or dissimilar modes of action. Ethinyl estradiol, genistein, the mixture with phytoestrogens and the mixture called Emix were expected to have estrogenic modes of action, and changes reflecting an enhanced mammary development were expected. The study on ethinyl estradiol was designed with mammary gland examination as primary aim, whereas the other studies were primarily designed for optimal evaluation of other specific endpoints and mammary tissues were dissected when possible. Thus, the papers in the present thesis include other endpoints that are not part of the results of this PhD but will be discussed in relation to the effects observed in mammary glands and female external genital malformations. An overview of the endpoints investigated in the studies performed in each paper is presented in table 2, and the compounds and dose levels investigated are presented in table 3.

The following experimental goals were defined for papers 1-5:

Paper 1: The aim was to identify sensitive parameters to a potent estrogenic compound, ethinyl estradiol, for analysis of female and male rat mammary gland whole mounts pup day (PD) 21-22. Several parameters covering the evaluation of the outgrowth, differentiation and the density of the mammary glands were evaluated.

Paper 2: The aim was to identify sensitive parameters to a potent estrogenic chemical, ethinyl estradiol, for analysis of female and male rat mammary glands from adult offspring at PD50-55 and PD90 in histological slides. Furthermore, the sensitivity of female external genital malformations to ethinyl estradiol was evaluated.

Paper 3: The aim was to study the mammary gland endpoints in whole mounts and histological sections in female and male rat offspring exposed to a xenoestrogen, genistein, or a mixture of dietary phytoestrogens. All chemicals studied are suggested to have an estrogenic mode of action.

Paper 4: The aim was to study the mammary gland endpoints in whole mounts and histological sections in female and male rat offspring exposed perinatally to an environmentally relevant mixture of pesticides suggested to be anti-androgenic.

Paper 5: The aim was to study the mammary gland endpoints in whole mounts and histological sections in female and male rat offspring exposed to various environmentally relevant mixtures of chemicals, covering both estrogenic (Emix) and anti-androgenic (Amix) chemicals as well as a mixture of both types of EDCs and paracetamol (TotalMix). Additionally, the medical compound, paracetamol, was studied.

Appendix 1: The aim was to investigate the variation in control values for some parameters evaluated in whole mounts of mammary glands from the Wistar rat strain routinely used in our laboratory and study the differences between strains (Wistar and Sprague-Dawley) and sub-strains (from another supplier) used in some of our studies.

Appendix 2 The aim was to identify the most sensitive method for evaluation of female genital malformations in rats and to study the sensitivity of this endpoint to environmentally relevant chemicals. Several parameters for evaluation of the genital malformations were evaluated to decide the most reliable and sensitive method to a potent estrogenic compound, ethinyl estradiol.

Moreover, several single compounds and mixtures of EDCs were investigated for their effects on the parameter.

3.1. Study design

In general, all studies performed in the present thesis were designed in a comparable way. Pregnant rat dams were exposed by gavage from 4 days after arrival (GD7) until the day before expected delivery. The day of expected delivery was called PD0. Exposure was resumed the day after delivery until PD13, 16 or PD22 (Table 3). Necropsy of offspring was performed on PD21-22, PD50-55 and PD90 or older. At necropsy, evaluation of female external genital malformations was performed measuring the urethral slit length. The 4^{th} mammary gland was dissected for whole mounting (PD21-22) and in paper 1 and 5, part of the 5^{th} mammary gland was dissected with the 4^{th} mammary gland for supplementing measurements in whole mounts (Figure 1 and 4). For histology, the 4^{th} mammary gland was dissected (PD50, 90 and/ or older); however, when the 4^{th} mammary glands were dissected for other purposes, the 2^{nd} mammary gland was dissected for histology. Details on the experimental design of the studies are described in papers 1-5.

Table 2: Overview of the endpoints evaluated in the papers and manuscripts included in the present thesis.

Paper (P), Appen-dix (A)	**Strain**	**Chemical(s)**	**Whole mounts (WMs) and genders evaluated PD21/22**	**Histology of mammary glands and age at evaluation**	**Female genital malfor-mations and age at evaluation**	**N (litter)**
P1	Wistar	Ethinyl estradiol	Female and male WMs	-	-	6-9
P2	Wistar	Ethinyl estradiol	-	Female and male mammary glands PD50/55 and 90	PD22, 50, 90	6-9
P3	Sprague-Dawley	Genistein 213 mg/kg or a mixture of phytoestrogens	Female WMs	Male mammary glands PND150. Female mammary glands PND135	-	14-15
P4	Wistar	Mixture of pesticides	Female and male WMs	Female and male mammary glands PD50	-	9-17
P5	Wistar	Amix, Emix paracetamol or TotalMix	Female and male WMs	Male and female mammary glands 10 and 13 months old, respectively	PD22, 50, 13 months	16-20
A1	Sprague-Dawley and Danish and Swedish Wistar	Control values from studies performed in papers 1-5 and historical studies	Female and male WMs	-	-	7-17
A2	Wistar	Ethinyl estradiol, Bisphenol A, Epoxiconazole, Emix, Amix, TotalMix or paracetamol	-	-	PD22, PD50, PD90	6-20

Emix: mixture of estrogenic chemicals, Amix: mixture of anti-androgenic chemicals, TotalMix: mixture of chemicals with various modes of action. PD: pup day. PND: post natal day. N: number of litters on PD1.

Table 3: Overview of chemicals and doses used in the studies performed in the papers and manuscripts included in the present thesis.

Paper (P)/ Appendix (A)	Chemical(s)	Doses	Exposure period	Origin of chemicals
P1	Ethinyl estradiol	0, 5, 15 and 50 µg/kg bw/day	GD7-PD22	Contraceptive pills
P2	Ethinyl estradiol	0, 5, 15 and 50 µg/kg bw/day	GD7-PD22	Contraceptive pills
P3	Genistein	213 mg/kg bw/day	GD7-PND13	Soy products
	Phytoestrogens (SECO, daidzeine, genistein, equol, formononetin, biochanin, enterodiol, coumestrol, enterolactone, matairesinol, lariciresinol and pinoresinol)	Mixture doses were high human dietary intake x1, x10, x100	GD7-PND13	Soy, nuts and other plant products
P4	Mixture of pesticides (epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone)	Doses were 8.3%, 17% or 25% of NOAEL, correspondding to 14.6, 29.2 or 43.8 mg/kg bw/day of the mixture	GD7-PD16	Pesticides – fruits and vegetables
P5	Amix: DBP, DEHP, vinclozolin, prochloraz, procymidone, linuron, epoxiconazole and p,p'-DDE	Mixture ratios were based on "high human intake" estimates x200 and x450	GD7-PD22	Plasticizer in wrapping and food containers (DBP and DEHP), pesticides (vinclozolin, prochloraz, procymidone, linuron and epoxiconazole) and pesticide metabolite (p,p'-DDE)
	Emix: 4-MBC, OMC, Bisphenol A and butyl paraben	Mixture ratios were based on "high human intake" estimates x200 and x450	GD7-PD22	UV filters (4-MBC, OMC), polycarbonate plastics and epoxy resin (Bisphenol A), cosmetics (BP)
	Paracetamol	Based on "high human intake" estimates. 360 mg/kg bw/day	GD13-19 and PD1-22	Analgesic and antipyretic medicine
	TotalMix: Amix + Emix + paracetamol)	Mixture ratios were based on "high human intake" estimates x100, x200, x450	GD7-PD22	As for Amix, Emix and paracetamol
A1	Controls – no exposure	-	-	-
A2	Ethinyl estradiol (from P1 and P2) and Amix, Emix, TotalMix and paracetamol (from P5)	As in P1, P2 and P5	GD7-PD22	As for P1, P2 and P5
	Bisphenol A	0.025, 0.25, 5, 50 mg/kg bw/day	GD7-PD22	Polycarbonate plastics and epoxy resin
	Epoxiconazole	15, 30 mg/kg bw/day	GD7-GD17	Pesticide – fruits and vegetables

SECO: secoisolarici resinol. DBP: di-n-butyl phthalate, DEHP: di-(2-ethylhexyl) phthalate, P,p'-DDE: pesticide metabolite. 4-MBC: 4-methyl-benzylidene camphor, OMC: Octyl methoxycinnamate. GD: gestation day, PD: pup day. PND: post natal day. NOAEL: no observed adverse effect level.

3.2. Methods applied for mammary gland examination

Mammary glands in whole mounts and histological slides were examined for effects of different EDCs to study which parameters may be expected to be affected by EDCs.

In the present studies, mammary glands were spread on glass slides and pressed with clamps and stained with alum carmine. In general, whole mounts of prepubertal glands were evaluated for ductal outgrowth, density and differentiation. The ductal outgrowth was evaluated by measuring the area covered by the mammary gland, longitudinal growth, transverse growth, distance to the lymph node and distance to the 5^{th} gland (Figure 4). The longitudinal growth was defined as the longest distance covered by the gland in the longitudinal axis of the animal. The transverse growth was defined as the longest growth perpendicular to the longitudinal growth. The distance to the lymph node was defined as the shortest distance between the mammary gland and the lymph node. The distance to the 5^{th} mammary gland was defined as the shortest distance between the 4^{th} and the 5^{th} mammary glands. An example of the measurement of the outgrowth parameters evaluated in the studies described in the present project can be found in figure 4. The density was given a score (1-5) according to the extension of branching and budding, with 5 being the most dense gland. The scoring scale was adjusted for gender, as male mammary glands generally are less developed compared to female mammary glands. The differentiation was evaluated by the number of TEBs.

Adult female and male mammary glands were evaluated in H&E stained histological slides at different ages in post pubertal offspring. In general, female mammary glands were evaluated in a specific stage of the estrous cycle and were evaluated for the extent of lobular development. However, for the studies with phytoestrogens, genistein and the mixture of pesticides (papers 3 and 4) females were sacrificed regardless of estrous cycle. The evaluation of lobuloalveolar pattern was distinguishable from the normal lobular changes due to the estrous cycle and was evaluated although females were not in the same stage of the estrous cycle. Male mammary glands were evaluated for tubuloalveolar pattern, secretory activity (vacuolated epithelium and secretory material in ducts and alveoli) and hypertrophy of mammary epithelium and the presence of apocrine-like changes in most of the studies.

The measurements of whole mounts and female urethral slit lengths were performed by Karen Riiber Mandrup or by other persons under supervision of Karen Riiber Mandrup. Histological examination was performed by Karen Riiber Mandrup.

3.3. Methods applied for female external genital malformation examination

In the literature, various methods for evaluation of female external genital malformations have been described. Appendix 2 describes how several parameters were measured in female external genitals exposed to a potent estrogenic chemical, ethinyl estradiol, to evaluate the most useful and sensitive parameter for evaluation of female external genital malformations. In further studies on environmentally relevant EDCs, external genital malformations in female offspring were evaluated measuring only the urethral slit length. The external genitals were evaluated in female offspring exposed perinatally to a potent estrogenic chemical, ethinyl estradiol, single compounds of the environmentally relevant chemicals bisphenol A, epoxiconazole or paracetamol or mixtures of environmentally relevant chemicals with Emix, Amix or TotalMix.

4. Results

In the following, an overview of the results and conclusions on whole mounts of mammary glands, histological slides of mammary glands and female genital malformations is described for each paper. Results from papers 1-5 are summarized in tables 4-8 according to the endpoints investigated. Results on whole mounts from PD21-22 are summarized in table 4 (females) and 5 (males). Results from histology of adult mammary glands are summarized in table 6 (females) and 7 (males). Results of female external genital malformations are summarized in table 8.

Paper 1: The aim was to identify sensitive parameters to a potent estrogenic chemical, ethinyl estradiol, for analysis of mammary gland whole mounts PD21-22. Twelve different parameters were evaluated in the whole mounts, covering parameters for ductal outgrowth, density and differentiation. Changes were seen from 15 µg/kg in females: a decrease in the relative distance to the lymph node and the distance to the 5th gland and an increase in the density were observed. In males, the number of TEBs and the density were increased at 50 µg/kg. Together, these changes confirm that the estrogenic chemical increases the growth of mammary glands and progresses the development of female and male mammary glands before puberty. The parameters sensitive to exposure to ethinyl estradiol PD22 were the distance to the lymph node, the distance to the 5th gland and the density in females, and in males PD21 the density and the number of TEBs.

Paper 2: The aim was to identify sensitive parameters to a potent estrogenic chemical, ethinyl estradiol, for analysis of mammary glands from adult offspring at PD50-55 and PD90 in histological H&E stained slides. Furthermore, the endpoint “female external genital malformations” was evaluated. No statistically significant changes were found in adult mammary glands, however, trends were observed in the same direction as effects reported in other studies. This lack of statistical significant changes may be due to a low power of scoring data and/or a shorter dosing period compared to other studies. An effect was, however, found on genital malformations in females. An increased prevalence of females had deep urethral slit length in the highest dose group (50 µg/kg) compared to controls PD50 and the urethral slit length was increased PD90 in the highest dose group.

Paper 3: The aim was to study the endpoints in rat mammary glands in whole mounts of prepubertal female offspring and in histological slides of adult female and male offspring after

perinatal exposure to “high human intake” of estrogenic chemicals originating from plant products (phytoestrogens) (Table 3). Genistein and a mixture of phytoestrogens showed no effects on the parameters evaluated in female whole mounts (area, longitudinal growth, transverse growth, density). Adult mammary glands showed an increased prevalence of hypertrophy of mammary epithelium in males PND150 exposed to phytoestrogens or genistein compared to controls. This increase was statistically significant in the highest dose of phytoestrogens. Moreover, an increase in vacuolated mammary epithelium and in the presence of secretory material in alveoli was observed in males and may be a sign of feminisation of the mammary glands, although not statistically significant ($p=0.06$). Females PND135 showed an increase in the prevalence of animals showing a lobuloalveolar pattern, indicating a masculinisation of the mammary glands, however, this increase was not statistically significant.

Paper 4: The aim was to study the endpoints in rat mammary glands in whole mounts of prepubertal offspring and histological slides of adult offspring after perinatal exposure to environmentally relevant anti-androgenic EDCs. A mixture of pesticides with suggested anti-androgenic mode of action (Table 3) showed no effects on the parameters investigated in whole mounts PD22 (area, longitudinal growth, transverse growth, density and number of TEBs). Histological evaluation of male mammary glands showed an increased prevalence of mammary glands with secretory activity (increased secretory material in alveoli and vacuolated mammary epithelium), suggesting a feminisation of the male mammary glands. Lobuloalveolar morphology of adult female mammary glands was observed in the exposed groups, however, this change was not statistically significantly different from controls.

Paper 5: The aim was to study the endpoints in whole mounts of prepubertal and histological slides of adult mammary glands of offspring exposed perinatally to three different mixtures of EDCs, Emix, Amix and TotalMix or to paracetamol (Table 3). A decrease in the distance to the lymph node was observed in females from the Emix and TotalMix groups. No effects were observed in offspring exposed to Amix or paracetamol. An effect on hypertrophy of male mammary epithelium may be present in the Emix group, however, a high prevalence was also seen in the control group and may be due to age-related changes and mask a possible dose-related effect. Females that had reached one year of age showed changes of extensive secretory activity and lobular development in the TotalMix group, suggesting an increased prevalence of prolactin producing pituitary tumors.

In the following, results for whole mounts of prepubertal mammary glands and histological evaluation of adult mammary glands, respectively, will be summarised, evaluating the results across papers 1-5 and appendix 1 and 2.

4.1. Whole mounts of prepubertal mammary glands

In whole mounts, female mammary glands PD22 were evaluated for ductal outgrowth (e.g. area, longitudinal growth, and distance to lymph node), density and differentiation (number of TEBs). Overall, estrogenic chemicals seemed to decrease the distance to the lymph node in females. Both the potent estrogen, ethinyl estradiol, the mixture of environmentally relevant estrogens (Emix) and the TotalMix (also containing estrogenic chemicals) affected the distance to the lymph node. It should be noted that the distance to the lymph node was not evaluated in offspring exposed to the mixture of phytoestrogens; and for ethinyl estradiol, the distance to the lymph node was evaluated as the growth towards the lymph node relative to the total distance between the nipple and the lymph node, but not as an absolute distance. Moreover, a decrease in the distance to the 5^{th} gland in offspring exposed to ethinyl estradiol confirmed the observed increase in outgrowth in this study. Ethinyl estradiol – as the only chemical studied – also increased the density of the female mammary glands. Anti-androgens, however, did not affect the parameters evaluated in whole mounts of female prepubertal mammary glands. Yet, the distance to the lymph node was not evaluated for the mixture of pesticides.

Table 4. Summary of results from paper 1-5 on whole mounts from female offspring PD22 exposed to endocrine disrupting chemicals perinatally.

PD22 females		Area	LG	TG	Distance to lnn	Distance to 5th gland	Density	TEBs	Paper
Ethinyl estradiol	5 µg/kg/day	-	-	-	-	-	-	-	1
	15 µg/kg/day	-	-	-	↓	↓	↑	-	
	50 µg/kg/day	-	-	-	-	↓	-	-	
Genistein	213 mg/kg/day	-	-	-			-		3
Phytoestrogens	1x	-	-	-			-		3
	10x	-	-	-			-		
	100x	-	-	-			-		
Emix	200x	-	-	-	↓	-	-		5
	450x	-	-	-	↓	-	-	-	
Pesticides	14.6 mg/kg/day	-	-	-			-	-	4
	29.2 mg/kg/day	-	-	-			-	-	
	43.8 mg/kg/day	-	-	-			-	-	
Amix	450x	-	-	-	-	-	-	-	5
TotalMix	450x	-	-	-	↓#	-	-	-	5
Paracetamol	360 mg/kg/day	-	-	-	-	-	-	-	5

x: fold human intake. LG: longitudinal growth. TG: transverse growth. lnn: lymph nodes. TEBs: terminal end buds. Emix: mixture of estrogenic chemicals. Amix: mixture of anti-androgenic chemicals. TotalMix: mixture of estrogenic and anti-androgenic chemicals and paracetamol. Pesticides: mixture of pesticides with proposed anti-androgenic effects. Grey areas represent parameters not evaluated for the chemical. Arrows: statistically significant increase or decrease compared to controls. #: statistically significant decrease when two similar studies were pooled. -: no statistically significant changes.

In male mammary glands, outgrowth was not affected by the EDCs studied. However, the density and the number of TEBs were increased by ethinyl estradiol. Thus, an increased branching and budding as well as a sign of increased proliferation were observed in male mammary glands after exposure to a potent estrogenic chemical. Overall, only ethinyl estradiol was able to affect male mammary glands in prepubertal whole mounts.

Table 5. Summary of results on whole mounts from male offspring PD22 exposed to endocrine disrupting chemicals perinatally.

PD22 males		Area	LG	TG	Distance to lnn	Distance to 5th gland	Density	TEBs	Paper
Ethinyl estradiol	5µg/kg/day	-	-	-	-		-	-	1
	15µg/kg/day	-	-	-	-		-	-	
	50µg/kg/day	-	-	-	-		↑	↑	
Emix	200x	-	-	-	-	-	-	-	5
	450x	-	-	-	-	-	-	-	
Pesticides	14.6mg/kg/day	-	-	-			-	-	4
	29.2mg/kg/day	-	-	-			-	-	
	43.8mg/kg/day	-	-	-			-	-	
Amix	450x	-	-	-	-	-	-	-	5
TotalMix	450x	-	-	-	-	-	-	-	5
Paracetamol	360 mg/kg/day	-	-	-	-	-	-	-	5

x: fold human intake. LG: longitudinal growth. TG: transverse growth. lnn: lymph nodes. TEBs: terminal end buds. Emix: mixture of estrogenic chemicals. Amix: mixture of anti-androgenic chemicals. TotalMix: mixture of estrogenic and anti-androgenic chemicals and paracetamol. Pesticides: mixture of pesticides with proposed anti-androgenic effects. Grey areas represent parameters not evaluated for the chemical. Arrows: statistically significant increase or decrease compared to controls. -: no statistically significant changes

4.2. Rat strain differences in mammary whole mounts

Results from control data on area, distance to the lymph node, density and number of TEBs in female and male mammary whole mounts are shown in appendix 1. Statistically significant differences in whole mount parameters were observed between strains but also between sub-strains and within the same sub-strain of Wistar rats. Control data from whole mounts of female and male mammary glands PD21-22 showed that Sprague-Dawley female rats have larger mammary glands compared to female Wistar rats. Wistar rats from different suppliers (different sub-strains) showed differences in the number of TEBs in females and males and in the mean density in males. Moreover, offspring from Wistar dams from the same sub-strain routinely used in our lab showed differences in area, density and number of TEBs.

4.3. Histology of adult mammary glands

An overview of the results of histological examination of adult mammary glands is shown in table 6 (females) and 7 (males). In females, no statistically significant changes were found. Lobuloalveolar pattern of the mammary glands is observed in rare cases. Such changes were only observed in exposed animals in the study with the mixture of pesticides and an increased prevalence was also seen after exposure to phytoestrogens. In those two studies only one control female showed lobuloalveolar pattern of the mammary glands. Moreover, enhanced lobular development was observed after perinatal exposure to ethinyl estradiol, a change not observed in the other studies. Lobule development is important for assessment of differentiation of the mammary glands. However, lobule development was not assessed for the studies with genistein, phytoestrogens and pesticides as females were killed at different stages of the estrous cycle in these studies.

Table 6. Histological findings in adult female mammary glands.

Study	Lobuloalveolar pattern	Lobules	Estrous cycle	n	Paper
Ethinyl estradiol PD90	-	(↑)	Diestrous	5-9	2
Genistein PND135	-		Varying	11-13	3
Phytoestrogens PND135	(↑)		Varying	11-15	3
Emix 13 months old	-	-	Estrous	11-16	5
Amix 13 months old	-	-	Estrous	13-16	5
Pesticides PD50	(↑)		Varying	9-14	4
Paracetamol 13 months old	-	-	Estrous	13-16	5
TotalMix 13 months old	-	-	Estrous	12-16	5

n: number of animals examined. PD: pup day. PND: post natal day. Emix: mixture of estrogenic EDCs. Amix: mixture of anti-androgenic EDCs. TotalMix: mixture of Emix, Amix and paracetamol. Parameters not evaluated a specific study are marked with grey cells. ↑: statistically significantly increase compared to controls ($p<0.05$). (↑): changes described and discussed in papers 2-5, although not statistically significantly different from controls

In males, an increased prevalence of hypertrophy was observed after exposure to estrogenic chemicals in offspring 90 days old or older. This was the only change observed in adult male mammary glands that was statistically significantly different from controls. In 10 months old males, a high prevalence of hypertrophic epithelium was present in controls. Secretory activity appeared to be increased by phytoestrogens and pesticides. Thus, both estrogenic and anti-androgenic chemicals may affect male mammary glands towards feminisation of the glands, although these changes are not consistent in all studies with estrogenic and anti-androgenic chemicals.

Table 7. Histological findings in adult male mammary glands.

Study	vacuoles	secretion	Hypertrophy	Apocrine-like changes	n	Paper
Ethinyl estradiol PD50	-	-	-	-	5-8	2
Ethinyl estradiol PD90	-	-	(↑)	-	8-10	2
Phytoestrogens PND150	(↑)	(↑)	↑	(↑)	12-15	3
Genistein PND 150	-	-	(↑)	-	12-13	3
Emix 10 months old	-	-	-	-	16	5
Amix 10 months old	-	-	-	-	15-16	5
Pesticides PND50	(↑)	(↑)			6-9	4
Paracetamol 10 months old	-	-	-	-	12-16	5
TotalMix 10 months old	-	-	-	-	13-16	5

n: number of animals examined. PD: pup day. PND: post natal day. Emix, Amix, TotalMix: as for table 6. Parameters not evaluated a specific study are marked with grey cells. ↑: statistically significantly increase compared to controls. (↑): changes described and discussed in papers 2-5, although not statistically significantly different from controls

4.4. Female genital malformations

As described in appendix 2, the urethral slit length was evaluated to be the most reliable parameter to investigate female external genital malformations after perinatal exposure to EDCs and this endpoint was sensitive in adult females PD90 exposed perinatally to a potent estrogen, ethinyl estradiol. The urethral slit length measured in offspring exposed perinatally to environmentally relevant chemicals showed no statistical significant changes. A summary of the results for urethral slit length from the present work is presented in table 8.

Table 8. Results on female external genital malformations.

Chemical	Doses	PD22	PD50	PD90	PD400
Ethinyl estradiol	5 µg/kg/day	-	-	-	
	15 µg/kg/day	-	-	-	
	50 µg/kg/day	-	↑	↑	
Bisphenole A	0.025 mg/kg/day	-		-	
	0.25 mg/kg/day	-		-	
	5 mg/kg/day	-		-	
	50 mg/kg/day	-		-	
Epoxiconazole	15 mg/kg/day	-		-	
	30 mg/kg/day	-		-	
Emix	200x	-	-		-
	450x	-	-		-
Amix	200x	-	-		-
	450x	-	-		-
TotalMix	100x	-	-		-
	200x	-	-		-
	450x	-	-		-
Paracetamol	360 mg/kg/day	-	-		-

x: fold "high human intake". Emix: mixture of estrogenic chemicals. Amix: mixture of anti-androgenic chemicals. TotalMix: mixture of estrogenic and anti-androgenic chemicals and paracetamol. Ages not evaluated for a specific exposure are marked with grey cells. ↑: Statistically significant increase in the urethral slit length ($p<0.05$).
- : no statistically significant changes in the urethral slit length.

4.5. Comparison with other EDC-sensitive endpoints

Other endpoints than mammary glands and female genital malformations may be more sensitive to EDCs, and other endpoints were evaluated in several of the studies described in the present PhD thesis. The low observed effect level (LOEL) for known estrogen- or anti-androgen-sensitive endpoints were compared with the LOEL for the endpoints studied in the present project.

Ano-genital distance (AGD) and nipple retention are endpoints known to be sensitive to anti-androgenic chemicals. Nipple retention was increased in male offspring in the studies on anti-androgenic pesticides, Amix and TotalMix [Christiansen et al. 2012; Hass et al. 2012]. Mammary gland effects of these mixtures were found in prepubertal females showing a decreased distance to the lymph node in the highest TotalMix dose. However, this effect was observed at a higher dose (TotalMix-450, i.e. 450 fold human exposure) than nipple retention (TotalMix-150, i.e. 150 fold human exposure) and the mammary gland effect may be contributable to the estrogenic chemicals in the mixture. Female genital malformations were evaluated for Amix and TotalMix, but no

changes were found. Thus, nipple retention was the most sensitive endpoint in the studies investigating the effects of anti-androgenic chemicals (Table 9).

Estrogen-sensitive female endpoints like sexual maturation and regularity of the estrous cycle were also evaluated. No statistically significantly effects of ethinyl estradiol, genistein, phytoestrogens or mixture of pesticides were found on these endpoints (papers 2, 3 and 4). Statistically significant effects on mammary glands were observed in offspring exposed to ethinyl estradiol or phytoestrogens. Female genital malformations were observed in the study with ethinyl estradiol. Thus, mammary glands and female genital malformations are female endpoints that appear to be affected by estrogenic chemicals and detect statistically significant effects when traditional female endpoints do not, although the number of animals studied was similar for all endpoints across a study.

The estrogenic chemicals ethinyl estradiol, genistein and the phytoestrogen mixture also showed effects on AGD in the highest dose-groups (papers 2, 3 and Christiansen et al., 2012). Data on nipple retention were present for ethinyl estradiol, only, and showed an increased number of nipples in females in the highest dose-group. However, mammary effects were observed at lower dose-levels than effects in AGD and nipple retention (Table 9). Mammary whole mounts showed increased outgrowth of female mammary glands defined by a decrease in the distance to the lymph node and distance to the 5^{th} gland at 15 µg/kg ethinyl estradiol and a decreased distance to the lymph node at Emix-200. The distance to the lymph node and the distance to the 5^{th} gland were not evaluated in the study with phytoestrogens and genistein. However, in that study, female mammary glands showed increased lobuloalveolar morphology in all phytoestrogen dose-groups, although not statistically significant. Moreover, hypertrophy of male mammary glands and female genital malformations were observed at the same dose levels as AGD and nipple retention in the study on phytoestrogens and ethinyl estradiol, respectively. Thus, outgrowth parameters, such as the distance to the lymph node, measured in mammary whole mounts in female offspring PD22 appears to detect effects of estrogenic chemicals at lower levels than AGD and nipple retention. However, AGD and nipple retention are more sensitive to anti-androgens than female genital malformations evaluated by the urethral slit length and mammary gland endpoints in whole mounts and histological examination.

Table 9. Statistical significant low observed effect level (LOEL) observed for each of the endocrine-sensitive endpoints mammary whole mounts, mammary histology, ano-genital distance index (AGDi) and nipple retention (NR) in the studies investigated in the present PhD thesis.

	Mammary whole mounts	**Mammary histology**	**Female genital malformations**	**AGDi or NR**
Ethinyl estradiol	**15µg/kg**: female distance to lnn, distance to 5th gland, density. 50µg/kg: male density and number of TEBs.	-	50µg/kg PD50 and PD90	50µg/kg: AGDi and number of nipples increased in females
Genistein	- (distance to lnn not determined)	-		AGDi decreased in males. (NR not determined)
Phyto-estrogens	- (distance to lnn not determined)	**x100**: Hypertrophy in males.		**x100**: AGDi decreased in males. (NR not determined)
Emix	**x200**: distance to lnn decreased in females	-	-	-
Pesticides	-	-		**14.6 mg/kg**: NR increased in males 29.2 mg/kg: AGDi increased in females *(1)*
Amix	-	-	-	**x450**: NR increased in males *(2)*
TotalMix	TotalMix-450: distance to lnn decreased in females	-	-	**x150**: NR increased in males *(2)*

x: fold high human intake estimates. lnn: lymph nodes. TEBs: terminal end buds. Emix: mixture of estrogenic chemicals. Amix: mixture of anti-androgenic chemicals. TotalMix: mixture of endocrine disrupting chemicals with dissimilar modes of action. Endpoints not determined for a specific exposure are marked with grey cells. The lowest dose with an observed effect (LOEL) for the compound or mixture is marked in bold red. 1: [Hass et al. 2012], 2: [Christiansen et al. 2012]. -: no statistically significant effects observed.

5. Discussion

Environmental EDCs were found to affect mammary gland development in female and male rats, with effects also detectable in adulthood. This stresses the possible influence of environmental chemicals on breast development in humans. In contrast, only a model compound, ethinyl estradiol, showed effects on female external genitals.

5.1. Effects of EDCs on mammary gland whole mounts

Investigation of mammary gland effects may be conducted in whole mounts or histological slides, in females or males, before or after puberty, in mice or rats. Mammary gland morphology has been widely studied at different ages in whole mounts from mice [Hilakivi-Clarke et al. 1997; Thomsen et al. 2006], Sprague-Dawley rats [Cotroneo et al. 2002; Fritz et al. 1998] and Long Evans rats [Hovey et al. 2010; Moon et al. 2007]. However, studies on whole mounts in Wistar rats are sparse, although Wistar rats are commonly used in toxicological studies. As reviewed by Rudmann et al. (2012), differences in susceptibility to neoplastic lesions are common between strains of rats [Rudmann et al. 2012]. Thus, differences may be expected between strains in the morphology of mammary glands and is important to investigate if mammary glands should be included in standard toxicological studies. In the present studies, differences between strains and sub-strains were present in prepubertal mammary gland morphology. However, effects of EDCs are detectable in mammary glands of rats in all strains investigated.

In the present studies, female mammary glands were affected by estrogenic chemicals, leading to increased density and increased outgrowth (decreased distance to the lymph node and for ethinyl estradiol a decreased distance to the 5^{th} gland), although changes in outgrowth were not detectable in the measurement of area, longitudinal growth or transverse growth. This is in accordance with previous studies showing altered mammary gland development after perinatal exposure to estrogenic chemicals. Increased outgrowth (increased area) [Murrill et al. 1996] and an increased number of TEBs [Cotroneo et al. 2002] were shown in prepubertal female rat offspring exposed to genistein postnatally. However, other studies with perinatal or postnatal exposure to estrogenic chemicals have not shown statistically significant effects in whole mounts of female prepubertal

rats or mice [Cabanes et al. 2004; Muñoz-de-Toro et al. 2005; You et al. 2002]. Thus, findings on estrogenic compounds are not consistent among studies. This may be due to the different parameters investigated and the different methods used to examine the parameters in whole mounts. Moreover, it may be due to the chemicals studied. A difference in potency of the compounds may be present and EDCs classified as estrogenic may also have other modes of action influencing the changes observed in mammary glands.

Few studies have investigated the effects of estrogenic chemicals in prepubertal male mammary glands, and male mammary glands have been reported to be more sensitive to EDCs compared to females [You et al. 2002]. You et al. (2002) showed statistically significant effects of genistein in whole mounts of male mammary glands PD22 and no effects in females [You et al. 2002]. In the studies performed in this PhD project, an increase in the density and the number of TEBs was found in male offspring after exposure to ethinyl estradiol but not to other estrogenic compounds. The increased density reflects an increase branching and budding of the glands and this finding is thus in accordance with the findings reported by You et al.. The number of TEBs was not changed in females and it was hypothesized that the number of TEBs in males may be more sensitive to estrogenic chemicals compared to females.

One study only could be found describing the effects of developmental exposure to an anti-androgenic chemical on the whole mounted mammary glands of pubertal rats. Saad et al. (2011) observed increased branching in whole mounts of pubertal (post natal day 35) female mammary glands following perinatal exposure to 1 mg/kg/day of the anti-androgenic pesticide vinclozolin [Saad et al. 2011]. No changes in outgrowth were reported in other studies investigating the effects of anti-androgens on whole mounts of pubertal mouse mammary glands [Peters et al. 2011; Skarda 2003]. In female mice, peripubertal exposure (week 5-12) to the anti-androgenic medicament flutamide did not show effects on ductal growth or branching [Peters et al. 2011]. In male mice, prepubertal exposure to the anti-androgenic medicaments flutamide, bicalutamide (Casodex) or chlormadinone acetate did not show effects on the area of the mammary glands [Skarda 2003]. In accordance with these findings, no change in outgrowth was observed in either male or female offspring PD22 exposed perinatally to anti-androgenic chemical mixtures (paper 4 and 5). The density was not changed with exposure, reflecting no observed effects on branching and budding PD22. The increased branching observed by Saad et al. (2011) may be explained by the higher dose or other modes of action of the chemical studied. Collectively, it appears that perinatal exposure to

anti-androgens does not affect mammary gland morphology prepubertally. However, anti-androgens may possibly cause other changes in mammary glands prepubertally, and morphological changes may be observable later in life as discussed later.

In prepubertal mammary glands of Wistar rats, no parameters appeared affected by anti-androgens, yet some parameters were identified to be sensitive to estrogenic chemicals. In females, the density of the female mammary glands was increased by ethinyl estradiol, indicating an increased branching and budding of the mammary gland. However, this effect was not found with other EDCs studied in this PhD project. This may be due to the potency of ethinyl estradiol compared to the EDCs studied. The distance to the lymph node and the distance to the 5th gland were outgrowth parameters that appeared sensitive to estrogenic chemicals although the area was not changed by exposure. The distance to the lymph node was decreased by ethinyl estradiol and Emix, whereas the distance to the 5th gland was only decreased by ethinyl estradiol. Thus, the distance to the lymph node appeared to be more sensitive to environmentally relevant estrogenic compounds compared to the distance to the 5th gland and area measurements. Vandenberg et al. (2007) showed that outgrowth parameters in mice may be influenced by the position of the foetus *in utero*. The gender of the sibling lying next to the offspring examined appears to affect branching, area and ductal elongation [Vandenberg et al. 2007]. A female offspring positioned between two females had less developed mammary glands (smaller area, ductal extension and fewer TEBs) compared to females positioned between two males [Vandenberg et al. 2007]. This may lead to a large variation in these parameters and thus a low power. A large variation in the area may explain why changes were not observed for this parameter when changes were seen in the distance to the lymph node. The same parameter – distance to the lymph node – was statistically significantly decreased in several groups of females exposed to estrogenic compounds: 50 µg/kg ethinyl estradiol, Emix-200 and Emix-450. Thus, the decrease in the distance to the lymph node may not be a Type I error (false positive effect due to) but may be a change due to exposure to estrogenic compounds. The decrease in this parameter indicates an increased growth of the female mammary glands towards the lymph nodes. Evaluation of whole mounts revealed that mammary glands in general grow primarily towards the lymph nodes (Figure 6) and ducts furthest away from the lymph nodes are poorly developed. Other parameters evaluated did not specifically determine the growth towards the lymph nodes and this may explain why other parameters like the transverse growth and area did not show significant changes, although the distance to the lymph node was significantly decreased. The ductal elongation from the nipple towards the lymph node may be possible to measure instead of the

distance between the edge of the mammary gland and the lymph node and may also be a parameter sensitive to estrogenic chemicals. However, this would decrease the number of measurements possible to perform, as the nipple is not always present in whole mounts and thus the power of the parameter would decrease. The positioning and size of the lymph nodes may differ between individuals and may influence the parameter. Knowledge on this aspect is necessary to investigate to determine the relevance of a changed distance to the lymph node. The sensitivity of the distance to the lymph node to estrogenic chemicals needs to be further investigated.

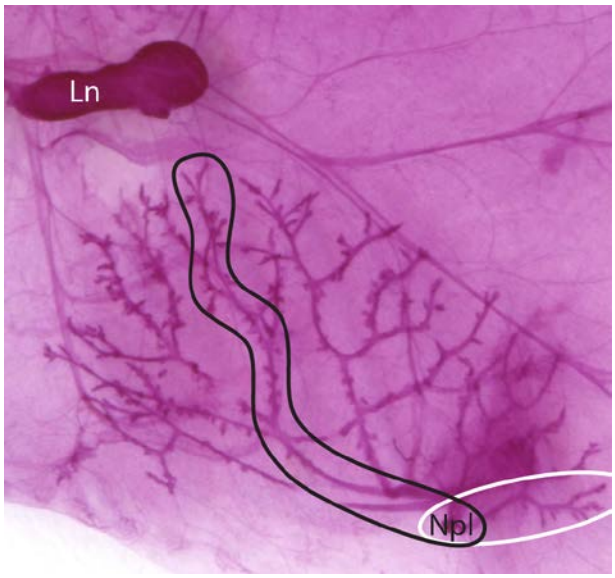

Figure 6. Growth of ducts in prepubertal female mammary glands. Mammary gland whole mount from a 22 days old control female rat. Ducts appear to grow primarily towards the lymph nodes (Ln) (a single duct is marked in black). Ducts furthest away from the lymph nodes are poorly developed (a single duct marked in white).
Npl: nipple.

In males, the density and the number of TEBs PD21 were parameters sensitive to the potent estrogenic chemical, ethinyl estradiol. The findings indicated a feminisation of the male mammary gland through an increased growth determined by an increased branching and budding of the gland, and an increased growth potential (TEBs) with an increased risk of development of mammary cancer. These changes were seen at 50 µg/kg, whereas changes in female whole mounts were observed from 15 µg/kg. Moreover, effects seen in males exposed to ethinyl estradiol were not found for other estrogenic EDCs studied. Thus female mammary glands appear to be more sensitive to estrogenic chemicals than male mammary glands when evaluating mammary glands in whole mounts. This is in contrast to histological examination of mammary glands as will be discussed later.

Overall, developmental exposure to estrogenic but not anti-androgenic chemicals appeared to have effects on the outgrowth and density in female mammary glands and on the density and number of TEBs in male mammary glands prepubertally. Evaluation of prepubertal mammary glands whole mount from pups exposed perinatally to potential EDCs should include the distance to the lymph node, the distance to the 5^{th} gland and the density in females and the density and the number of TEBs in males. Further insights into effects on mammary glands of compounds with other modes of action are needed.

5.2. Strain differences in mammary gland whole mounts

As shown in appendix 1, difference in mammary gland morphology was present between Wistar sub-strains from different suppliers and between strains (Wistar and Sprague-Dawley) and care should be taken in comparing crude numbers between studies using different strains or sub-strains. However, a difference in control values between sub-strains and strains of rats may not indicate a lower sensitivity of the endpoint. Other endpoints in rats are known to differ in control values between strains but are sensitive endpoints used in toxicological studies, e.g., litter size and body weight. In mammary glands, it is well known that some strains are more prone to develop mammary tumors than other strains as reviewed by Rudmann et al. (2012) [Rudmann et al. 2012]. For toxicological studies it is important to be aware of such differences. Mammary glands may be sensitive to endocrine disruption even though differences between strains and sub-strains exist. Although no effects were observed in prepubertal mammary glands of Sprague-Dawley rats exposed to phytoestrogens (paper 3), other studies with Sprague-Dawley rats have shown effects of EDCs in whole mounts around PD22. Several studies have shown statistically significant more TEBs in 21 days old females exposed postnatally to estrogenic compounds like bisphenol A, genistein, estradiol benzoate or flaxseed [Cotroneo et al. 2002; Moral et al. 2008; Tan et al. 2004]. Additionally, effects of EDCs on mammary gland morphology have been reported in Long Evans rats. Atrazine, an anti-androgenic compound, was shown to delay the migration to the lymph node and fewer primary branches were observed in mammary glands of 22 days old female offspring [Moon et al. 2007]. Wistar offspring have also been shown to be sensitive to EDCs. Ethinyl estradiol increased the density in females and males, decreased the distance to the lymph node in females and increased the number of TEBs in males (paper 1). Moreover, a decreased distance to

the lymph node was found in females exposed to a mixture of environmentally relevant estrogenic chemicals (Emix) (paper 5). Overall, the migration to the lymph node, density and number of TEBs are parameters that have been shown to be affected by EDCs. To my knowledge, a difference in sensitivity of whole mount examination between strains and sub-strains has not been investigated in the literature, and further studies may elaborate on this point.

5.3. EDC effects on mammary gland histology

In the present studies, changes in adult mammary glands were observed in offspring exposed to estrogenic chemicals. Adult females exposed to ethinyl estradiol showed increased lobular hyperplasia compared to controls and the mixture of phytoestrogens increased the prevalence of lobuloalveolar pattern. In adult males exposed to the estrogenic chemicals ethinyl estradiol, phytoestrogens or genistein, an increase in the prevalence of animals with hypertrophic mammary epithelium was observed in the present studies. These findings are in accordance with the findings reported by others [Delclos et al. 2001; Masutomi et al. 2004; Takagi et al. 2004]. Moreover, signs of increased secretory activity were seen in males. Secretory vacuoles and dilated ducts have also been reported in adult male mammary glands exposed to estrogenic chemicals [Biegel et al. 1998; Latendresse et al. 2009]. Secretory activity is a feature specific for female mammary glands, and may reflect a feminisation of the gland. Estrogenic feminisation of male mammary glands showing increased tubuloalveolar pattern has been reported previously for ethinyl estradiol and 17β-estradiol [Andrews et al. 2002; Biegel et al. 1998]. Tubuloalveolar morphology is the typical morphology in female mammary glands and the appearance of tubuloalveolar morphology in males is also perceived as a sign of feminisation of the male mammary glands.

Developmental exposure to a mixture of anti-androgenic pesticides (paper 4) also showed signs of increased secretory activity of adult male mammary glands. However, no changes were observed in the adult mammary glands in the study investigating the effects of environmentally relevant anti-androgens of various origins (Amix) (paper 5). Adult female offspring exposed to the mixture of anti-androgenic pesticides showed lobuloalveolar pattern in 3 exposed animals compared to none in the control group (paper 4). Although few animals showed such changes they may be exposure-related as discussed later. To my knowledge, only one study has investigated the effects of an anti-

androgenic chemical on the histology of adult mammary glands. Lee et al. (2004) observed increased incidence of vacuolar degeneration in alveolar cells and alveolar atrophy in the mammary glands of adult males (PD50) exposed perinatally to the anti-androgenic chemical di-*n*-butyl phthalate [Lee et al. 2004]. Thus, it can be hypothesized that anti-androgens affect adult male mammary glands. Androgens contribute to male differentiation of mammary glands to a lobuloalveolar pattern and it may be hypothesized that perinatal exposure to anti-androgens changes the responsiveness of male mammary glands to androgens later in life. Changes observed in females exposed to pesticides were similar to changes observed in females exposed to phytoestrogens and may be due to other modes of action of the chemicals.

Most of the changes described in the present studies were not statistically significant. The only histological finding that was statistically significantly changed compared to controls was the increase in hypertrophy in male mammary glands in the study with phytoestrogens (paper 3). However, other changes may be related to exposure, although not statistically significant. Lobuloalveolar pattern of the female mammary gland PD90 and 135 is a rare finding in control animals. Only one control female among 33 control females examined before the age of 1 year showed a lobuloalveolar pattern in the present studies. In the study with phytoestrogens, lobuloalveolar pattern was observed in 11 of the exposed females. Such findings in exposed groups may be an important sign of change due to exposure, although not statistically significant. In general, histological evaluation has low power compared to quantitative data, as the data from such analyses are scoring data or dichotomous in nature [OECD 2010a]. Low power of histological scoring data calls for a large number of animals to detect statistical significant changes. The small number of animals studied in some of the present papers, may thus be responsible for the lack of significant effects and trends in histological findings investigated.

None of the changes observed were possible to determine as attributable to a specific group of chemicals with similar modes of action. EDCs often have combined modes of action and the EDCs studied do not have purely estrogenic or purely anti-androgenic modes of action. The differences in effects observed may be explained by the different properties of the compounds studied, but also by a difference in age at evaluation of the mammary glands. In the study with mixtures of environmentally relevant chemicals (paper 5), mammary glands were evaluated approximately at PD300 (males 10 months old) and PND400 (females 13 months old). Around this age some control female rats are beginning to achieve reproductive senescence and pituitary tumors arise

spontaneously in some rats. As a result, evaluation of female mammary glands at this age may show various changes reflecting changes either related to hormonal changes due to reproductive senescence or pituitary tumors. Such changes may mask changes related to exposure. Examination of exposure-related morphological changes in female rat mammary glands should thus preferably be evaluated before age-related changes occur in controls. However, other changes, e.g., neoplastic transformation and tumors, may be relevant to study after reproductive senescence. Age is also an important consideration to keep in mind for examination of male mammary glands, in which differentiation and maturation occurs after sexual maturation. In the present studies, a change in hypertrophy of the epithelial cells was only apparent in male mammary glands PD 90 and 150 (paper 2 and 3). However, in 10 months old males, the prevalence of males with hypertrophic epithelium in offspring exposed to the estrogenic chemicals (Emix) was at the same level (81%) as in the younger males (88% of males PD90 exposed to ethinyl estradiol and 80% of males PD150 exposed to phytoestrogens), but with a higher control-level of hypertrophy (60%) at 10 months of age. Thus, 10 months old males may demonstrate the same morphology as young males exposed to estrogenic chemicals. However, in 10 months old males hypertrophic mammary epithelium was more common in controls (paper 5) and a change in exposed animals compared to controls was not detectable. Hypertrophy may be a change naturally occurring with age in male mammary epithelium. Estrogenic chemicals may accelerate this change. It is encouraged to evaluate female and male mammary glands before signs of aging appear in controls, but the exact age when signs of aging show up in controls, has not been determined. Moreover, in accordance with findings reported by others [Ahren and Etienne 1957; Sourla et al. 1998], male mammary glands PD50 had not yet fully differentiated to lobuloalveolar pattern in the present studies. Changes like feminisation of the male mammary glands reflected by tubuloalveolar morphology or secretory activity is more evident in males where male differentiation has occurred and the typical male mammary architecture is present. Rat mammary glands are preferably evaluated after gender-specific differentiation and before signs of aging has occurred.

Overall, perinatal exposure to EDCs affects mammary gland development in female and male offspring and changes are observable histologically in adults. Female mammary glands may show changes in lobuloalveolar pattern and lobular hyperplasia. Male mammary glands may show changes in hypertrophy of mammary epithelium and signs of feminisation, such as secretory activity or tubuloalveolar morphology. Trends in histological changes are important to investigate for parameters like the lobuloalveolar pattern that seldom appear in controls. Rat mammary glands

should be evaluated after sex-specific morphological differentiation has occurred and before age-related changes may occur. Strain differences may also be present in the timing of maturation of mammary glands. According to the present studies, Wistar rats should in general be evaluated around PD90, but further studies are necessary to determine the best time-points for Wistar rats as well as for other stains. Other mammary changes such as neoplastic transformation and tumors may be relevant to study in older rats.

5.4. Sensitivity of mammary gland endpoints

In the present project, mammary glands were examined in prepubertal and sexually mature female and male rats. Evaluation of whole mounts of prepubertal mammary glands can show effects on outgrowth and differentiation before puberty and give a good overview of the gland *in toto*. In contrast, histological evaluation of adult mammary glands can show epithelial changes as well as differentiation of the gland. Estrogenic chemicals have been shown to affect both prepubertal and adult mammary glands. The present study on ethinyl estradiol (paper 1 and 2) showed statistically significant effects in whole mounts of prepubertal mammary glands and no statistically significant effects in adult mammary glands. In accordance, two papers reported results in mammary glands of male offspring exposed prepubertally to genistein. In those studies, statistically significant effects were found in parameters evaluated in whole mounts whereas no statistical results were reported for the histological changes observed [Wang et al. 2006; You et al. 2002]. Evaluation of prepubertal mammary glands in whole mounts appeared more sensitive to detect statistically significant effects of developmental exposure to estrogenic chemicals compared to the evaluation of histological sections of adult mammary glands. Only trends to dose-dependent histological changes were shown in histological examination of the adult glands. In contrast, the study on phytoestrogens (paper 3) showed statistically significant changes in histology of adult male mammary glands, yet, no changes were observed in the mammary whole mounts of prepubertal offspring. Thus, histological examination of adult male mammary glands appeared more sensitive to detect changes from phytoestrogens than evaluating whole mounts prepubertally. This difference in detectability of effects of estrogenic chemicals may be due to different properties of chemicals. As noted, statistically significant effects may in general be more easily detected in whole mounts compared to histological evaluation of the mammary glands due to low power of histological data. However,

changes that are not statistically significant should not be ignored and quantitative methods for histological examination could be useful.

Gender-specific differences in sensitivity were observed in prepubertal as well as adult mammary glands. Morphological analysis of whole mounts of prepubertal mammary glands in the study on ethinyl estradiol (paper 1) showed a statistically significant increase in the number of TEBs PD21 in male mammary glands at 50µg/kg. No effect in the number of TEBs in females was observed, suggesting that the number of TEBs is a parameter more sensitive to estrogenic chemicals in male mammary glands than in females. In accordance with these findings, You et al. (2002) showed more marked effects in branching in whole mounts of 22 days old male mammary glands than in females [You et al. 2002]. However, in the present study, female mammary gland whole mounts showed a statistically significant change in outgrowth not seen in males. A decrease in the distance to the 5th gland was seen at 15µg/kg in offspring exposed to ethinyl estradiol and the distance to the lymph node was decreased in female offspring exposed to ethinyl estradiol or the Emix (paper 1 and 5). However, the distance to the 5^{th} gland was not changed in females in other studies with estrogenic compounds and the decrease found in the study with ethinyl estradiol may be a chance finding. Nevertheless, the outgrowth (distance to the lymph node) appeared more sensitive in females than in males and may be a parameter more sensitive than the number of TEBs to estrogenic chemicals. However, it should be noted that the distance to the 5th gland was not possible to measure in males and it is not known whether female and male mammary glands differ in sensitivity regarding this endpoint.

In histological examination of adult mammary glands, a more marked effect was observed in male mammary glands as compared to females. In the study with phytoestrogens (paper 3), male mammary histology showed statistically significant changes in the highest mixture dose-group whereas no statistically significant changes were observed in female mammary histology in adult offspring. Other studies have shown a similar difference between male and female mammary gland sensitivity to exposure to EDCs. In studies with perinatal exposure to genistein, histopathological examination of mammary glands showed hyperplasia in male mammary glands at a lower dose than changes observed in female mammary glands [Delclos et al. 2001]. Similarly, other studies on genistein or ethinyl estradiol showed hyperplasia of male mammary glands when no histological changes were reported in females [Andrews et al. 2002; Wang et al. 2006]. This may point to a higher sensitivity of male than female mammary glands. Males are known to have lower levels of

estrogen compared to females and male mammary glands may thus be more sensitive to small changes in the estrogen levels, e.g., caused by low levels of estrogenic EDCs.
Collectively, this may indicate that male mammary glands are more sensitive to EDCs than female mammary glands in respect to the development of TEBs and histological changes, whereas female outgrowth of prepubertal mammary glands is more sensitive to estrogenic compounds compared to male outgrowth. In the case of the study with phytoestrogens, a lower NOAEL for mammary gland effects was seen when not only female, but also male mammary glands were evaluated. Thus, it is important to include evaluation of male mammary glands in addition to females, as also reflected in recent updates of OECD guidelines for reproductive toxicity [OECD 2010b].

Overall, histological examination of mammary glands may show changes that should not be neglected, although not statistically significant. Moreover, male mammary glands appear sensitive to EDCs and should be included in the investigation of effects of EDCs in vivo.

5.5. Female external genital malformations

As described in appendix 2, several methods for evaluation of female external genital malformations were investigated in a study with ethinyl estradiol. The distance to the vaginal opening was technically difficult to assess. Thus, the urethral-vaginal distance and the relative urethral slit length were not reliable measurements unless the person performing the measurement is well trained and experienced in the procedure. The urethral slit length and the evaluation of the presence of a clefted genital papilla were sensitive measurements that were the most reliable.
Two studies by Sawaki et al. (2003a and b) showed an increased urethral slit length in adult female offspring exposed perinatally to 50 µg/kg ethinyl estradiol. Accordingly, the present study showed increased urethral slit length in females PD90 exposed to 50 µg/kg ethinyl estradiol. However, offspring PD22 and 50 did not show changes in the urethral slit length, suggesting that changes in the urethral slit length are not as marked in young animals. This is in contrast to Flaws et al. (1997) reporting macroscopically visible clefts at the time of vaginal opening (PND28-40) [Flaws et al. 1997]. This may be due to a difference in the compound studied. Flaws et al. (1997) investigated the effects of 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), an endocrine disrupting compound with

other modes of action, and may be more potent than ethinyl estradiol on influencing the development of female external genitals [Heiden et al. 2008].

Effects on female genital malformations have been reported for different compounds, such as TCDD, ethinyl estradiol, tamoxifen and testosterone propionate, with various endocrine disrupting modes of action, suggesting effects of a wide range of EDCs on female external genitals [Flaws et al. 1997; Gray et al. 1997; Gray and Ostby 1995; Wolf et al. 1999a; Wolf et al. 2002; Yamasaki et al. 2005]. However, in the present studies on environmentally relevant EDCs, no changes in the urethral slit length were shown. No genital malformations in females exposed perinatally to the Emix were observed, although changes were seen in females exposed to the potent estrogenic compound, ethinyl estradiol. This may be due to a difference of potency and the dose level of Emix selected.

5.6. Human relevance of EDC effects on mammary glands and female external genitals

Estrogenic compounds were shown to increase outgrowth of rat mammary glands, suggesting an early breast development in girls exposed to estrogenic chemicals during foetal development and the lactation period. As noted, precocious puberty and breast development may increase the risk for breast cancer later in life [Pike et al. 1981]. Thus, perinatal exposure to EDCs with estrogenic mode of action may indirectly lead to breast cancer in women. It should be noted that dose-levels used in some of the studies are very different from human exposures. High doses were used in the studies on ethinyl estradiol and pesticides (papers 1, 2 and 4), whereas the studies on phytoestrogens and contaminants (papers 3 and 5) used doses with a lower safety margin. Further risk assessment is required for further determination of human risk, but this is out of scope of this thesis.

Developmental exposure to EDCs led to feminisation and hypertrophy of male adult mammary glands. Other studies have shown feminisation of sexually mature male mammary glands by EDCs [Andrews et al. 2002; Biegel et al. 1998]. Such changes may suggest development of gynecomastia in men after puberty. However, this relation is yet to be verified.

An increased number of TEBs in male mammary glands and female genital malformations were observed in offspring exposed to a potent estrogen, but not in other studies with environmentally relevant EDCs. These changes may suggest an increase in the risk for mammary cancer and a

disturbed development of the external genitals in females by potent estrogens. Human exposure to the mixtures of EDCs in the environment does not appear to be at levels with risk for such changes.

5.7. Regulatory relevance

Mammary gland development and genital malformations in female rats were investigated to determine if these female endpoints were affected by the selected EDCs. The endpoints were evaluated to determine if they were able to detect effects of EDCs.

As described in section 4.5, AGD and nipple retention were endpoints more sensitive to anti-androgens than female genital malformations evaluated by the urethral slit length and mammary gland endpoints in whole mounts and histological examination. However, prepubertal female mammary glands detected effects at lower levels of perinatal exposure to estrogenic chemicals than other endpoints sensitive to EDCs. The distance to the lymph node measured in mammary whole mounts in female offspring PD22 detected effects when other estrogen-sensitive endpoints, such as sexual maturation and irregularity of estrous cycles, did not. Moreover, the distance to the lymph node was affected by estrogenic chemicals at lower levels than AGD and nipple retention.
Male mammary glands also showed effects of estrogenic chemicals. The number of TEBs PD21 and hypertrophy of the mammary epithelium were affected in adult male rats (Table 9), effects not detected in female rats. These effects may reflect changes of importance for the risk for mammary cancer or possibly gynecomastia in men. However, the observed effects may also be relevant for female mammary glands, but appeared to be more sensitive parameters in male rat mammary glands compared to female rats.

Histological examination of adult but not prepubertal mammary glands is included in the extended one-generation studies [OECD 2010b]. This type of study is used for approval of pesticides and biocides in the European Union, but not for other types of chemicals. The two-generation study is the reproductive study used for other chemicals and mammary gland examination is not included in this study [OECD 2001]. Thus, only pesticides and biocides are investigated for mammary gland effects.

Inclusion of prepubertal female and male rat mammary glands in toxicological studies is important as effects of EDCs may go undetected if only adult rat mammary glands are studied. Some features of prepubertal rats make examination of such mammary glands easier compared to histological examination of adult mammary glands, and evaluation of male mammary whole mounts is somewhat easier than the evaluation of female mammary glands. In adults, evaluation of mammary glands of females is dependent on estrous cycle for some parameters [Schedin et al. 2000]. The lack of an estrous cycle in prepubertal females facilitates the timing of necropsy and the evaluation of the gland. Male mammary glands are less developed than female mammary glands and have fewer TEBs than females. Thus, counting of TEBs PD21 may be faster in male mammary glands compared to females.

Adverse effects of chemicals on mammary glands may be overlooked and estrogenic effects detectable in mammary glands may not be observed when prepubertal and adult mammary glands are not investigated in the guideline studies.

5.8. Further studies

Scoring data are known to have low power and although changes may be present, a statistical analysis may not necessarily detect a change. Quantitative measurements are more sensitive than scoring data and quantitative methods are preferable to use. Computerised methods may help to quantitate morphological features such as the density in whole mounts, lobular development or hypertrophy in histological slides. For example, the number of branch points or the number of terminal ends in mammary gland whole mounts may be determined by an image analysis programme. In histological slides, the area of the most developed lobules may be possible to measure as well as the area or intensity of the staining of cells and nuclei. Such parameters may give a quantitative measure for the density in whole mounts and for the differentiation (lobular development) or hypertrophy in adult mammary glands. Few studies have described advanced methods for quantitative image analysis of rodent mammary glands [Fernandez-Gonzalez et al. 2004; McGinley and Thompson 2011], but more simple techniques should be developed to facilitate the implementation of the endpoints in guideline studies.

Histological examination of male mammary glands in the studies on phytoestrogens and environmental relevant EDCs (paper 3 and 5) showed hypertrophic changes resembling apocrine changes described in humans [Fuehrer et al. 2012]. In humans, malignant and benign apocrine changes can be distinguished by immunohistochemical staining for androgen receptors (AR), estrogen receptors (ER) and progesterone receptors (PR) [Gatalica 1997; O'Malley 2004]. In the present studies apocrine-like changes were observed in controls and exposed rats (papers 3 and 5) and the biological relevance of these changes in rodents is unknown. Further studies may clarify if the apocrine-like changes observed in rodents are comparable to the human apocrine changes and if it is possible to distinguish between benign and malignant types by using immunohistochemical staining as in humans. Moreover, other effects in the mammary glands may be relevant to investigate by immunohistochemical staining (for example staining for proliferation or apoptosis) or gene expression of the mammary glands.

6. Conclusions

The present project aimed at verifying the hypothesis that (i) exposure to EDCs during foetal and prepubertal development affects mammary gland development in rats, (ii) female external genital development in rats is altered by EDCs and (iii) changes in female or male mammary glands or female genital malformations may be good predictors for endocrine disrupting effects of a chemical.

Mammary gland development in rats was affected by perinatal exposure to EDCs. Although differences in whole mounts of control mammary glands were present in Sprague-Dawley and Scanbur Wistar rats compared to Taconic Wistar rats, the parameters were sensitive to EDCs. EDCs with estrogenic mode of action appeared to increase mammary outgrowth in prepubertal female rats and a potent model compound, ethinyl estradiol, increased the density in females and males and the number of TEBs in male rats. Moreover, phytoestrogens showed changes in epithelial morphology in male (hypertrophic epithelium) and female (lobuloalveolar morphology) mammary glands in adult rats. Anti-androgenic chemicals showed signs of feminisation of adult male mammary glands. No effects of anti-androgens were observed in female mammary glands.

Female external genitals in rats were affected by a potent estrogenic model compound, ethinyl estradiol, however, no effects were found in females exposed to the environmentally relevant estrogenic chemicals bisphenol A, epoxiconazole or compounds in the Emix. No effects of anti-androgens were observed in female external genitals. Estrogenic compounds may perturb development of female genitals, however, this was not seen for the EDCs and dose levels studied in the present project.

Overall, female and male mammary glands were affected by a model compound (ethinyl estradiol) or environmentally relevant EDCs in the commonly used Wistar rats as known for other strains of rats. The changes observed in mammary glands and female external genitals may be of importance for human health. The present findings suggest that perinatal exposure to estrogenic EDCs may lead to precocious breast development in girls exposed to estrogenic compounds perinatally if exposure levels to these compounds are sufficiently high. Moreover, EDCs may lead to changes in adult male mammary glands, and this may indicate a role of EDCs in development of gynecomastia in men.

Ethinyl estradiol showed effects in female external genitals indicating a failure in the fusion of the urogenital folds and suggests a disturbance of the foetal development. Such disturbance may affect

other aspects in foetal development and is also of concern for humans and wild animals if exposure to potent estrogenic chemicals is present.

Outgrowth of prepubertal female mammary glands measured as the distance to the lymph node was observed at lower dose-levels of estrogenic chemicals compared to other endpoints known to be sensitive to EDCs. However, further studies are needed to confirm the sensitivity of the distance to the lymph node in whole mounts of female mammary glands to estrogenic compounds. Estrogenic effects on the number of TEBs in males and the density in females and males PD21-22 also appear to be relevant to investigate further.

The extended one-generation reproductive toxicity study described in the OECD guidelines for testing of chemicals (TG 443) includes sacrifice of female and male rats around PD90 [OECD 2010b]. At this age, histological mammary gland examination is included. Evaluation of female external genital malformations described in the present studies, can be performed, but may not be affected by estrogenic EDCs. However, in such a study, risk assessment of estrogenic chemicals may overlook the effects on mammary glands if outgrowth in females PD22 is not investigated. Thus, for evaluation of the effects of chemicals suspected to have endocrine disrupting effects, mammary glands of both male and female offspring may be relevant to evaluate, both prepubertally and in adults, using whole mounts and histology.

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PAPER 1

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Perinatal ethinyl oestradiol alters mammary gland development in male and female Wistar rats

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Summary

Increased attention is being paid to human mammary gland development because of concerns for environmental influences on puberty onset and breast cancer development. Studies in rodents have showed a variety of changes in the mammary glands after perinatal exposure to endocrine disrupting chemicals, indicating progressed development of mammary glands when exposed to oestrogens early in life. However, laboratories use different parameters to evaluate the development of mammary glands, making studies difficult to compare. Moreover, studies of whole mounts in Wistar rats are lacking. In the present study, Wistar rats were exposed to 0, 5, 15 or 50 μg/kg of ethinyl oestradiol per day during gestation and lactation. A wide range of morphological parameters were evaluated in whole mounts of mammary glands from male and female offspring PD21–22. This study showed that in both male and female pre-pubertal Wistar rats, mammary gland development was accelerated after perinatal oestrogen exposure with increase in size, density and number of terminal end buds (TEBs). In female rats, the most sensitive parameters were the distance to the fifth gland, the relative growth towards the lymph node and the overall density. The sensitive endpoints in male rats were TEB numbers, both in the whole gland and in the zone C, the overall- and the highest density. The overall density was sensitive in both male and female rats and was considered a good representative of both branching and budding of the gland. The number of TEBs in zone C was representative of the number of TEBs in the whole gland. Further studies in older Wistar rats and with weak oestrogenic compounds could be performed to validate mammary gland examination as an endpoint in reproductive toxicity studies and to examine how early life environmental exposures may alter mammary gland development, disrupt lactation and alter susceptibility to breast cancer.

Introduction

In the USA and Europe, the mean age of puberty onset in girls has decreased over the last decades, including early development of breasts (Mouritsen *et al.*, 2010). Early puberty onset increases the risk for breast cancer (Pike *et al.*, 1981; Medina, 2005; Rudel *et al.*, 2011), raising the concern for this decrease in age of puberty in the western world. Exposure to endocrine disrupting chemicals (EDCs) are suspected to be involved in the changes in puberty onset and breast development, and more attention is being paid to the effects of EDCs on pregnant women and their offspring, including the effects on mammary glands.

In rats, exposure to certain EDCs has been shown to alter both the timing of sexual maturation and the development of mammary glands (Hilakivi-Clarke *et al.*, 1997; Goldman *et al.*, 2000; Cotroneo *et al.*, 2002; Hovey *et al.*, 2005; Thomsen *et al.*, 2006). These changes are seen when rats are exposed prenatally, postnatally or both pre- and postnatally, showing that the mammary glands are sensitive to EDCs during a wide time span of the development of the animal. The mammary glands continue developing throughout life, and studies have shown that the glands

are especially sensitive to exposure around three phases where development is extensive in females. The first phase is the prenatal period where the mammary epithelial sprout is developed and the primary ducts grow into the fat pad. The second period is the pre-pubertal period where the mammary glands grow extensively with the outgrowth of several secondary and tertiary branches, occupying an increasing area of the fat pad, including growing of buds. The third period is during pregnancy, where the glands develop lobules and turn into milk producing glands (Fenton, 2006). Studies are still being performed trying to discern the specific time of development sensitive to EDCs in utero (Rayner *et al.*, 2005).

In the reproductive stage of life between puberty and menopause (excluding lactation), the mammary gland is a dynamic structure changing with the female cycle (Anderson *et al.*, 1998; Schedin *et al.*, 2000; Hvid *et al.*, 2010). In women, proliferation of the breast tissue is elevated in the mid-luteal phase of the menstrual cycle when the level of oestradiol peaks together with progesterone (Anderson *et al.*, 1998). Overall, several studies suggest that the hormone being the main mitogen in the breast tissue in both humans and rodents is oestradiol, possibly in combination with progesterone (Anderson *et al.*, 1998; Cotroneo *et al.*, 2002; Clarke, 2003; Gompel *et al.*, 2010). This encourages the theory that EDCs with oestrogenic properties can affect mammary glands and enhance mammary development and that exposure to EDCs may be involved in changes linked to early breast development. Exposure to EDCs before puberty may also affect the mammary glands and give rise to permanent changes and alter their susceptibility to environmental influences later in life. Foster *et al.* showed that although mammary glands did not show histopathological changes after exposure to EDCs in utero, an increased sensitivity to a repeated exposure to EDCs postnatally resulted in histopathological changes in mammary glands of the adult rat (Foster *et al.*, 2004).

Although several laboratories have examined changes in mammary glands in humans, rats and mice, no standard evaluation techniques have been agreed. Different structures are being evaluated and many different measurements are being performed (Brody *et al.*, 2011; Rudel *et al.*, 2011). Grossly, mammary glands of rodents are either evaluated in whole mounts or histologically. Laboratories evaluating whole mounts look at (i) the ductal development, for example by counting the number of primary ducts, measuring the ductal elongation, the length of the ductal tree along its longitudinal axis, the migration of the mammary epithelium towards the lymph node, area occupied by the parenchyma or the outermost edges of the epithelial tree or the distance between neighbouring glands; (ii) the gland differentiation and alveolar development by counting or giving a score for the amount of structures like terminal end buds (TEBs), terminal ducts and alveolar buds and lobules and (iii) the density of the mammary tissue by scoring the extension of development according to criteria relevant for the specific age and gender (Fielden *et al.*, 2002; Rayner *et al.*, 2004; Muñoz-de-Toro *et al.*, 2005; Moon *et al.*, 2007; Moral *et al.*, 2008; Hovey *et al.*, 2010). Moreover, mammary gland whole mounts are evaluated at different time points of development, including juvenile mammary glands before puberty and adult glands (Hilakivi-Clarke *et al.*, 1997; Hovey *et al.*, 2005; Thomsen *et al.*, 2006). These different measurements represent effects on different developmental processes: longitudinal growth of the ducts, increased branching, growth of buds and lobules or a combination. Mammary gland development is dependent on TEBs, undifferentiated structures at the end of the ducts, most distant to the nipple. They have a high proliferation rate, and are responsible for ductal outgrowth and give birth to new ducts and alveoli. The number of TEBs reaches a peak around post-natal day (PND) 20 and decreases at older age (Russo *et al.*, 1979). TEBs are the structures sensitive to carcinogens and give rise to mammary cancer (Russo *et al.*, 1979; Russo & Russo, 1996b; Fenton, 2006). Hence, chemically induced increase in the number of TEBs could be linked to an increased risk of mammary cancer (Fenton, 2006) and thus, TEBs are the main structure investigated in studies of mammary glands. As the TEBs are located at the end of the ducts, they are to be found in high numbers in the area of the gland furthest away from the nipple, called the zone C (Russo & Russo, 1996a). Yet, the rat mammary gland is a three-dimensional structure and TEBs could also be found in the other areas of the gland. Thus, it is of interest to investigate if TEBs should preferably be counted in the whole mammary gland of rats.

Oestrogenic chemicals have been shown to accelerate the development of female mammary glands. For example, studies with pups exposed during lactation with oestrogenic compounds in female mice showed increased branching, ductal criss-crossing and number of TEBs with 17β-oestradiol (Hilakivi-Clarke *et al.*, 1997; Thomsen *et al.*, 2006) and increased ductal outgrowth with diethylstilbestrol (DES) (Hovey *et al.*, 2005). In female pups of Sprague–Dawley (S–D) rats, similar changes were found with increased branching and number of TEBs when exposed to genistein, oestradiol benzoate or DES during the lactational period (Cotroneo *et al.*, 2002; Ninomiya *et al.*, 2007). In contrast, anti-oestrogenic compounds were found to have the opposite effect with fewer TEBs in offspring of dams exposed during lactation (Cotroneo *et al.*, 2002).

Few laboratories have studied the effects of EDCs in male mammary glands. Nonetheless, male mammary

glands from S–D rats are also sensitive to oestrogenic chemicals like genistein and ethinyl oestradiol (You *et al.*, 2002; Latendresse *et al.*, 2009). Later in life, even more prominent changes can be observed in male mammary glands (Wang *et al.*, 2006). Some chemicals may not have adverse effects on female mammary glands, but lead to developmental changes in male mammary glands (You *et al.*, 2002). It is desirable to detect these changes, as the incidence of breast cancer in men has been rising over the last decades in the US and UK (Hodgson *et al.*, 2004; Speirs & Shaaban, 2009). Moreover, changes seen in male rat mammary glands exposed to EDCs may reflect changes connected to breast cancer in men. Furthermore, an effect of EDCs on male mammary gland development may serve as a marker of endocrine disruption.

Several laboratories study the effects of oestrogenic chemicals on mammary gland whole mounts in mice (Hilakivi-Clarke *et al.*, 1997; Hovey *et al.*, 2005; Thomsen *et al.*, 2006), Sprague-Dawley (Cotroneo *et al.*, 2002; Ninomiya *et al.*, 2007) or Long-Evans rats (Moon *et al.*, 2007), but to our knowledge, investigation of the morphology of mammary glands in whole mounts in Wistar rats exposed to oestrogens is lacking. However, Wistar rats are widely used in toxicological studies, hence more knowledge about mammary gland changes in Wistar rats is desirable. In the current study, we hypothesize that Wistar rat mammary gland development is sensitive to oestrogenic chemicals and shows similar effects to those seen in mice and other strains of rats. The aim of this study was to investigate which endpoints in mammary gland whole mount analysis are sensitive to perinatal exposure to an oestrogenic model compound, ethinyl oestradiol. Several studies have shown histological changes such as hyperplasia of the mammary epithelium in S–D offspring 50 days old or older after maternal exposure to 50 ppb (approximately equivalent to 5 μg/kg) and 50 μg/kg of ethinyl oestradiol during gestation and after birth (Masutomi *et al.*, 2001; Takagi *et al.*, 2004; Latendresse *et al.*, 2009). However, other studies have shown increased foetal/neonatal morbidity in Long-Evans Hooded rats after exposure to 50 μg/kg/day of ethinyl oestradiol (Howdeshell *et al.*, 2008). Hence, 50 μg/kg/day was chosen as the highest dose in the present study and the lower dose levels of 5 and 15 μg/kg/day were chosen to gain knowledge of the dose–response curve. The whole mount method was evaluated in 21–22 days old male and female Wistar rats. To cover a wide span of sensitive periods in the development of mammary glands sensitive to EDCs, rats in this study were exposed during the majority of pregnancy (GD7–21) and from birth to necropsy on pup day (PD) 21–22. Sinha *et al.* showed that TEBs reach the maximum number from the third week of age up to PND 35, and many laboratories have found effects of EDCs on mammary glands PD 21 (Sinha *et al.*, 1983). Mammary glands from pre-pubertal female and male offspring were mounted as whole mounts and evaluated to reveal possible gender specific differences in sensitivity to oestrogenic-acting chemicals perinatally. A wide range of parameters were evaluated to compare different parameters for measuring changes in the mammary gland development and to find the most sensitive endpoints representing each of the three developmental types, (i) ductal development, (ii) gland differentiation and (iii) density.

Materials and methods

Experimental design

In this study, 40 time-mated nulliparous young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy. On the day after arrival (GD 4), the dams were distributed into four groups of ten animals to obtain similar weight distributions in each group. The animals were housed in pairs until GD 18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 × 27 × 43 cm) with Aspen bedding (Tapvei; Brogaarden, Gentofte, 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 lux, temperature 21 ± 2 °C, humidity 50 ± 5%, ventilation eight 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 was provided ad libitum. The dams were dosed by gavage from GD 7 to the day before expected birth (GD 21) and from PD 1 until PD 21 or 22. The control group was dosed with vehicle (corn oil; Sigma, Brøndby, Denmark) and the exposure groups were given 5, 15 or 50 μg/kg bw/day of ethinyl oestradiol (CAS 57-63-6 from Steraloids, nr. E1550-000). The expected day of delivery, GD 23, was designated PD 1. 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 of the National Food Institute at the Technical University of Denmark.

Mammary gland whole mounts

A total of 1–3 pups per gender per litter were weighed, anaesthetized and decapitated for necropsy. Male pups were necropsied on PD21 and female pups on PD22. The fourth abdominal mammary glands were dissected at necropsy including the nipple and the lymph node. The tissue was spread onto glass slides and covered with parafilm while drying for minimum 2 h, then fixed, stained with alum carmine, dehydrated and mounted.

Whole mounts were scanned in a flatbed scanner (4800 dpi) and measurements were performed on the digital images in Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). Spatial calibration was performed for each picture before measurements were made, and data produced were exported to a Microsoft Excel spreadsheet. The glandular densities were scored by visual inspection of the digital images. The number of TEBs was determined by evaluation of the whole mounts under a light microscope at 5–10 times magnification. Buds were determined as TEBs if they had a diameter ≥100 μm as described by Brown *et al.* (Brown *et al.*, 1998). The single experimenter doing the morphometric analyses was blinded to the dose-groups. A parameter was not measured on the whole mount if the part of the gland important for the parameter was missing.

Several measurements were performed for each mammary gland. First, seven different parameters were measured representing the (i) ductal outgrowth: (1) number of primary ducts, (2) longitudinal growth (mm), (3) transverse growth (mm), (4) area occupied by the epithelial tree (mm^2), (5) outline area (mm^2) determined by the outline of the mammary gland, (6) when the fifth mammary gland was present, the distance between the fourth and fifth mammary gland was measured and (7) relative growth to the lymph node assessed as the percentage of the total distance from the nipple to the lymph node covered by mammary tissue. Next, three different parameters were measured representing the (ii) gland differentiation: (8) score of number of lateral buds (1–5), (9) number of TEBs in zone C, (10) number of TEBs in the whole gland. Finally, two different densities were achieved taking into account the evaluation of both budding and branching development with a score from 1 to 5, with 1 representing few branches and buds, and 5 representing highly branched glands with many buds: (11) overall density defined as an average of the density of the whole gland, (12) highest density in the gland assessed as the highest density present in the gland (Fig. 1). Most of these parameters are used in other

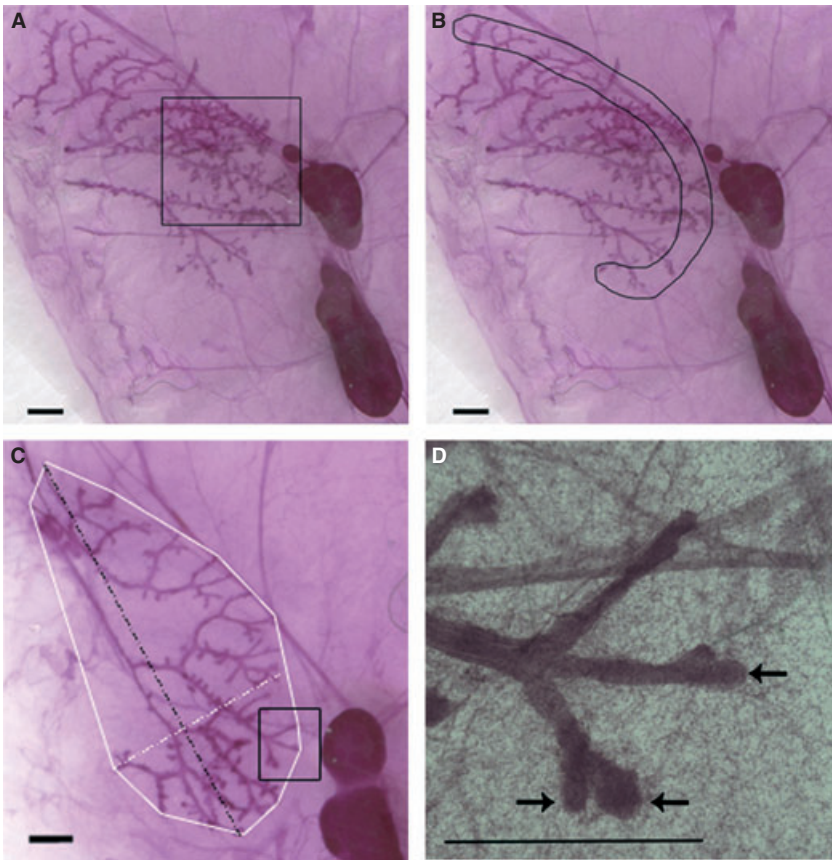

Figure 1 Female mammary gland whole mounts PD22. (A) Mammary gland with overall density score 3 and highest density 4 (box). (B) Mammary gland with zone C outlined. (C) Mammary gland with the parameters 'outline area' (white polygon), 'longitudinal growth' (black dotted line) and 'transverse growth' (white dotted line) depicted. The black box is enlarged in d. (D) Mammary gland viewed in a microscope with magnification 5×. TEBs are shown (arrows). Bars in the lower left corner of each picture represent 1 mm. The dark areas in the lower right corner of pictures a, b and c are lymph nodes.

laboratories to evaluate mammary gland whole mounts. However, the relative growth to the lymph node is a new alternative to measure the ductal outgrowth taking into account the size of the animal without using the body-weight (bw). Studies have shown that ethinyl oestradiol decreases offspring neonatal bw and weight gain until PND 21 (Masutomi *et al.*, 2001; Takagi *et al.*, 2004). Thus, a measurement independent of the bw is desirable. The two means of counting TEBs were performed to compare the relationship between the number of TEBs in the most distant part of the gland (zone C) (Fig. 1) and the TEBs in the whole gland. The measurement 12, 'highest density', is an alternative to the overall density scoring as some glands are unequally dense, and it may be of interest to discern if a mammary gland is affected with higher density in any part of the gland (Fig. 1). As mammary glands from control female and male rats are slightly different, a different scale for density scoring was used for male and female mammary glands to be able to differentiate even small changes in the male mammary glands.

Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Statistical analysis of variance (ANOVA) was performed using SAS Enterprise Guide 3.0 statistical software (SAS Institute Inc, Cary, NC, USA). Correlation and linear regression analysis were performed in Graph Pad Prism 5. Discrete data were analysed with a one-way ANOVA (Mixed Models) with bw as a covariate and litter as a random factor. However the bw was analysed without a covariate. The relative growth to the lymph node is a measure taking into account the size of the animal, and the statistical analysis was consequently performed without bw as a covariate. The litter means of the mammary gland densities were evaluated for treatment effects using a one-way ANOVA with heterogeneous variance. Residuals were verified for homogeneity of variance and normal distribution before performing the statistical tests. The number of TEBs in zone C and in the whole gland in male mammary glands varied from 0 to 2, except for few animals with more TEBs. Therefore, a supplementary Fisher's exact test was performed for the male TEBs. The TEBs in zone C and TEBs in the whole gland were tested for correlation within each dose group with a two-tailed Pearson analysis.

Results

In this study, some of the time-mated animals were not pregnant. Thus, the number of litters in the control and the two highest dose groups varied between seven and nine, and the lowest dose group had six litters. All control dams gave birth on GD23. One dam in the lowest dose-group and five of eight pregnant dams in the highest dose-group gave birth on GD24.

Female mammary glands

Mammary gland whole mounts in Wistar rats exposed to ethinyl oestradiol perinatally were evaluated for (i) ductal development, (ii) gland differentiation and (iii) density using several different measurements (Tables 1 and 2). In the female rats, the ductal development showed no significant changes in any group for number

Table 1 Female mammary gland evaluation PD 22 after in utero and lactational exposure to 0, 5, 15 or 50 μg/kg/day of ethinyl oestradiol. Mean of litter ± SD

Female PD22	Control	5 μg/kg	15 μg/kg	50 μg/kg
Body weight (g)	42.3 ± 4.5	39.0 ± 2.2	37.6 ± 4.3	38.0 ± 5.7
Number of primary ducts	2.5 ± 0.5	2.7 ± 0.6	2.7 ± 0.7	2.61 ± 0.5
Longitudinal growth (mm)	11.0 ± 1.2	10.6 ± 0.9	11.0 ± 1.6	10.7 ± 1.1
Transverse growth (mm)	6.0 ± 1.2	6.2 ± 1.1	6.2 ± 1.1	6.9 ± 1.5
Tree area (mm^2)	26.4 ± 12.6	27.8 ± 4.7	31.1 ± 5.3	34.2 ± 8.9
Outline area (mm^2)	40.9 ± 13.2	46.9 ± 8.5	47.1 ± 9.3	46.1 ± 9.7
Distance to fifth gland (mm)	10.3 ± 0.7	10.6 ± 1.3	7.8 ± 1.2[a]	7.6 ± 1.0[a]
Relative growth to the lymph node (%)	83.8 ± 7.8	87.8 ± 7.6	96.1 ± 6.5[a]	88.1 ± 9.4
Bud density (score)	3.0 ± 0.7	3.0 ± 0.7	3.3 ± 0.7	3.5 ± 1.1
TEBs in whole gland	23.6 ± 16.7	14.9 ± 19.4	32.1 ± 17.7	26.7 ± 12.4
TEBs in zone C	10.1 ± 10.7	7.0 ± 10.1	13.4 ± 9.6	12.9 ± 5.8
Overall density (score)	2.6 ± 1.1	2.5 ± 1.1	3.6 ± 0.7[a]	3.4 ± 1.1
Highest density (score)	3.1 ± 0.7	3.0 ± 1.0	3.8 ± 0.7	3.5 ± 1.0

[a]Statistically significantly different from controls, alpha level at $p < 0.05$. $N = 4$–8 litters, 1–3 pups per litter.
PD, pup day; TEBs, terminal end buds.

Table 2 Male mammary gland evaluation PD 21 after in utero and lactational exposure to 0, 5, 15 or 50 μg/kg/day of ethinyl oestradiol. Mean of litter ± SD

Male PD21	Control	5 μg/kg	15 μg/kg	50 μg/kg
Body weight (g)	39.4 ± 4.2	39.5 ± 5.5	36.8 ± 5.3	34.4 ± 4.8
Number of primary ducts	2.1 ± 0.7	2.0 ± 0.0	1.9 ± 0.5	2.0 ± 0.0
Longitudinal growth (mm)	10.2 ± 1.3	10.6 ± 1.3	9.4 ± 1.5	9.6 ± 2.0
Transverse growth (mm)	5.2 ± 0.9	5.6 ± 0.2	5.2 ± 0.9	5.2 ± 0.9
Tree area (mm^2)	14.5 ± 4.4	18.1 ± 4.7	12.4 ± 6.6	14.3 ± 6.1
Outline area (mm^2)	46.0 ± 8.6	45.9 ± 7.9	37.3 ± 15.8	37.0 ± 14.3
Relative growth to the lymph node (%)	77.4 ± 6.8	75.3 ± 10.9	83.3 ± 10.9	86.2 ± 10.3
Bud density (score)	1.6 ± 0.7	1.8 ± 0.3	1.9 ± 0.7	2.8 ± 1.6
TEBs in whole gland	0.7 ± 0.4	0.6 ± 0.9	1.2 ± 1.5	7.2 ± 7.3[a]
TEBs in zone C	0.6 ± 0.4	0.6 ± 0.9	0.9 ± 1.2	3.8 ± 4.0[a]
Overall density (score)	1.9 ± 0.6	1.9 ± 0.2	2.2 ± 0.7	2.8 ± 1.0[a]
Highest density (score)	2.3 ± 0.5	2.1 ± 0.5	2.6 ± 0.7	3.3 ± 1.1[a]

[a]Statistically significantly different from controls, alpha level at $p < 0.05$. N = 4–9 litters, 1–3 pups per litter.
PD, pup day; TEBs, terminal end buds.

of primary ducts, longitudinal growth and transverse growth (Table 1). The measurements of the area occupied by the mammary gland were increased with dosing, yet not statistically significant for either the outline of the glandular area or the tree area. The distance to the fifth gland was significantly shorter than controls in the two highest dose-groups. An attempt was made to make measurements independent of the bw using the relative growth to the lymph node. This measurement was significantly higher than controls for rats given 15 μg/kg ethinyl oestradiol. The gland differentiation showed a trend of an increased number of TEBs, however no statistically significant differences in the bud density, the number of TEBs in zone C (TEBC) or TEBs in the whole gland (TEBall) were present. When comparing TEBC and TEBall, the number of TEBC increased with the number of TEBall (Fig. 2). Data showed a clear correlation in all the dose-groups ($r^2 > 0.8$). The overall density and highest density of the glands appeared increased by oestradiol exposure, but this was only statistically significant for the overall density in the group dosed with 15 μg/kg ethinyl oestradiol (Table 1, Fig. 3). The bw of the female offspring PD22 appeared slightly decreased with dosing, but this was not statistically significant.

The measurements tree area and the number of TEBs in the zone C had a high variation in the controls, with standard deviations (SD) equal to the half of the mean or more (Table 1). The high variation was because of a single animal in the control group with more than five times more TEBs (34) compared with the mean of the rest of the controls (6.1 ± 2.2). Similarly, the mean and variation of the tree area was also increased because of this animal, which can be considered an outlier within normal biological variation.

Male mammary glands

Male mammary glands PD 21 were evaluated for the same endpoints as the female mammary glands (Table 2).

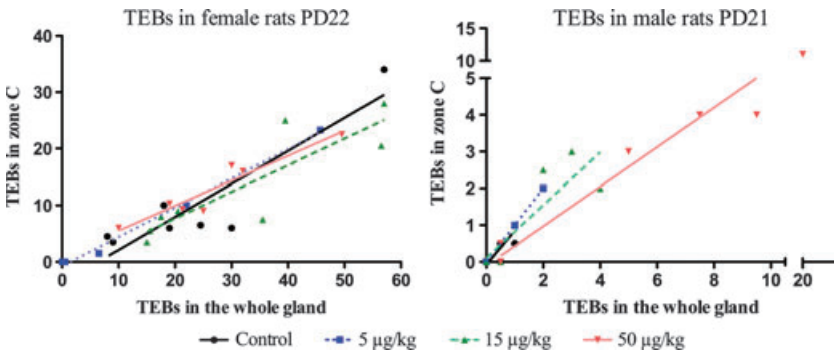

Figure 2 Correlation between the number of TEBs in the zone C and in the whole mammary gland in female and male Wistar rats PD21–22 after exposure to ethinyl oestradiol perinatally. Each data point represents the mean number of TEBs of a litter. The axes are broken in the figure for the male TEBs to make the correlations of the three lowest dose–groups visible.

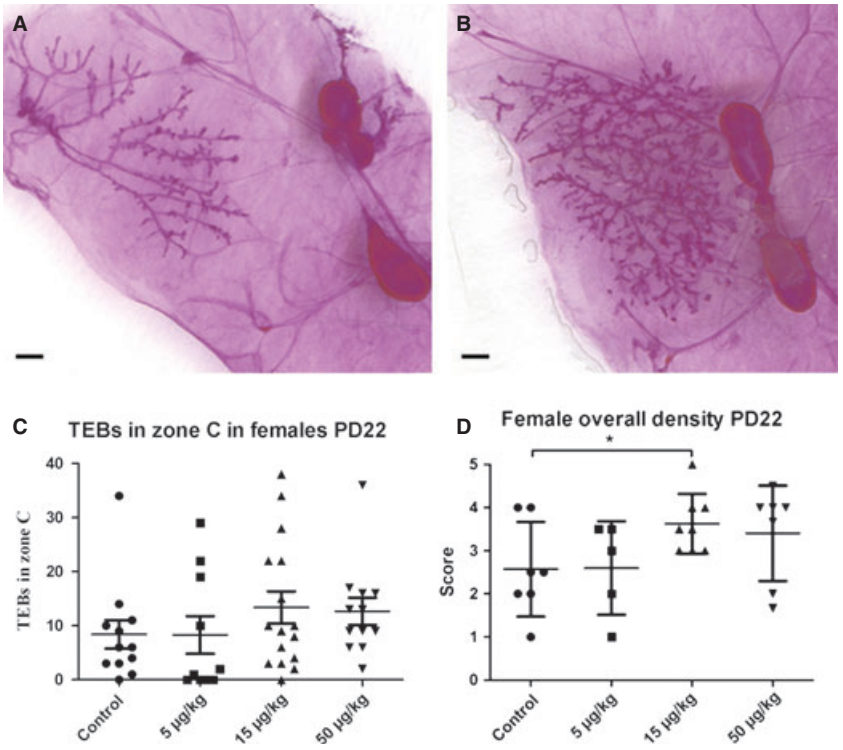

Figure 3 Female mammary gland density and terminal end bud (TEB) number PD22.(A) Mammary gland of control rat with density score 2. (B) Mammary gland of female rat exposed to 15 µg/kg of ethinyl oestradiol with density score 4. (C) TEB number in female mammary glands. Number of TEBs was increased with dosing, but the increase was not statistically significant. (D) Mean densities ± SD. Each point represents the mean density of a litter. Density was statistically significantly increased in females exposed to 15 µg/kg of ethinyl oestradiol ($p < 0.05$) (*). Bars in pictures a and b represent 1 mm.

The fifth mammary gland was only present in five of the whole mounts; hence this parameter is not shown. When examining the other measurements for ductal development, no statistical significant changes were seen in the mammary glands. For the gland differentiation, a statistically significant increase was only found for both the number of TEBs in the whole gland and the number of TEBs in the zone C at 50 µg/kg compared with control (Fig. 4). The number of TEBs varied from 0 to 2 in controls and rats exposed to 5 µg/kg/day of ethinyl oestradiol, independently of the zones (Fig. 2). TEBC and TEBall correlated well in all dose groups ($r^2 > 0.7$) (Fig. 2). With regard to the densities, both density measurements were significantly higher in the highest dose group (Fig. 4). The decrease in bw of male pups PD21 was not statistically significant.

Discussion

Pups in this study were exposed to ethinyl oestradiol perinatally to evaluate whether mammary gland examination in prepuberty, which is known to be sensitive to oestrogenic compounds in mice and certain strains of rats, is also sensitive to a potent oestrogen in Wistar rats.

Female mammary gland whole mounts PD22 revealed increased branching and budding expressed by an increased overall density of the mammary glands in animals exposed to 15 µg/kg/day of ethinyl oestradiol perinatally. Increased growth of branches was also shown by increased relative growth towards the lymph node at 15 µg/kg/day and a decreased distance to the neighbouring fifth gland at the doses 15 and 50 µg/kg/day.

In male mammary glands, the current study showed a statistically significant increase in TEB numbers and density in rats exposed to 50 µg/kg/day of ethinyl oestradiol compared with controls. This confirms the expectations that perinatal exposure to oestrogenic compounds progresses prepubertal development of mammary glands in males and females (Cotroneo *et al.*, 2002; You *et al.*, 2002).

Based on the present findings, the most sensitive endpoints in pre-pubertal female Wistar rats exposed to ethinyl oestradiol perinatally seem to be: the distance to the fifth gland, the relative growth to the lymph node and the overall density of the gland. In comparison, endpoints

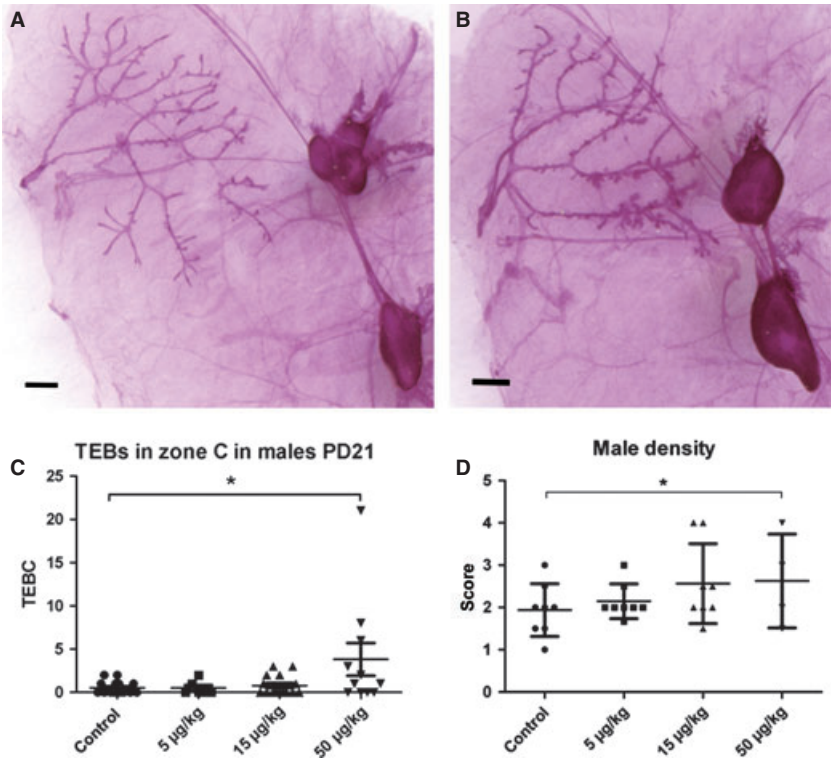

Figure 4 Male mammary gland density and terminal end bud (TEB) number PD21. (A) Mammary gland of control rat with density score 2. (B) Mammary gland of male rat exposed to 50 µg/kg of ethinyl oestradiol with density score 3. Notice the presence of more buds in picture B than A. (C) TEB number in male mammary glands. Number of TEBs was statistically significantly increased in rats exposed to 50 µg/kg of ethinyl oestradiol ($p < 0.05$) (*). (D) Mean overall densities + SD. Each point represents the mean overall density of a litter. Overall density was statistically significantly increased in males exposed to 50 µg/kg of ethinyl oestradiol ($p < 0.05$) (*). Bars represent 1 mm.

most sensitive in male Wistar rats seem to be: the number of TEBs in the whole gland, the number of TEBs in the zone C and the two means of measuring the density of branches and buds. Regarding the TEBs, the correlation between TEBC and TEBall, show that TEBC increases similarly when TEBall increases in both male and female Wistar rats. Hence, TEBC is a good parameter representing the number of TEBall, making this endpoint fast and easy to achieve. In female rats, a tendency to increased TEB numbers (in whole gland or region C) was not statistically significant. For females, data for the tree area and the number of TEBs in zone C showed a high variation in all groups. The control group had a mean number of TEBs in zone C of 10 and a SD of 11 [coefficient of variance (CV) = 110%]. A single animal in the control group was responsible for the high variation. Thus, changes in the tree area and the number of TEBs in zone C may have been more difficult to detect in the present study because of this animal. In a study by Moral *et al.* (2008) the number of TEBs in zone C in control animals is approximately 30 ± 5 (mean ± SEM) (read-out from graph in Fig. 1, Moral *et al.*, 2008), leading to a SD of 15 ($n = 9$) and a CV of 50% (Moral *et al.*, 2008). Other studies on S–D rats showed a similar variation in the number of TEBs PND 21. Cotroneo *et al.*, 2002 and Tan *et al.*, 2004 showed 34 ± 3 and 43 ± 5 (mean TEBs ± SEM), respectively, leading to a CV of 26.6% for Tan *et al.*, 2004 (Cotroneo *et al.*, 2002; Tan *et al.*, 2004). Thus, the variation in the number of TEBs in zone C in female rats is expected to be high, and the variation in the present study is further increased because of one animal.

TEBall and TEBC numbers varied between zero and two in most male mammary glands. For the TEBall, only 2 of 14 and 4 of 11 animals had more than two TEBs in the groups treated with 15 µg/kg and 50 µg/kg of ethinyl oestradiol respectively. Hence, a Fisher's exact test was run. However, some animals in the group exposed to 50 µg/kg of ethinyl oestradiol had noticeable more TEBs (more than 10). This number of TEBall was increased more than fivefold, and it was considered important to take this into account in the statistical test together with the litter. Thus, an ANOVA with bw as a covariate and litter as a random factor was run in addition to the Fisher's exact test. Both the ANOVA and the Fisher's exact test

showed the same result for TEBall, i.e. a statistically significant increase in the group exposed to 50 μg/kg of ethinyl oestradiol. It should be noted that with this heterogeneous type of data, it is important to select appropriate statistical analyses.

As the variation in the number of TEBs in female rats is high, only major changes in the number of TEBs can be detected. However, a low variation is observed in the number of TEBs in male mammary glands. Hence, this endpoint is more sensitive in male mammary glands. These findings are in accordance with a study by You *et al.*, 2002 (You *et al.*, 2002). In that study, mammary glands were evaluated from male and female pups of dams exposed via feed to the oestrogenic compounds genistein or methoxychlor at 300 or 800 ppm (equally to 15–30 and 40–80 mg/kg) in the diet throughout pregnancy and lactation until necropsy at PND 22. Similar to the current study, You *et al.* found an increase in the number of TEBs in male mammary glands, but not in female mammary glands. These findings suggest that male mammary glands indeed are more sensitive to oestrogen than female mammary glands regarding the number of TEBs. On the other hand, results from the present study suggest that the relative growth to the lymph node is a more sensitive endpoint to oestrogenic chemicals in female rat mammary glands than in males.

In the present study, the statistical tests for the area and distance to the fifth gland, includes bw measurements to take the size of the animal into account. However, the bw may reflect both the size of the animal but also the condition of the animal being either thin or overweight. Measurements independent of the bw are desirable, and other laboratories have also tried to make such measurements using the outline tree area relative to the fat pad area (Muñoz-de-Toro *et al.*, 2005). Laboratories have also measured the distance to the lymph node, but they have made the measurement in mm or given a score (Muñoz-de-Toro *et al.*, 2005; Moon *et al.*, 2007). In the current study, the distance is assessed as the migration towards the lymph node as a percentage of the total distance from the nipple to the lymph node. Hereby, this measurement is also independent of the bw. Further studies on this endpoint may be needed to validate this endpoint and resolve whether this is indeed more sensitive in female rats than male rats.

As mentioned earlier, mammary glands are sensitive to EDC exposure during several and long periods of the development of the animal. In the current study, animals were exposed to ethinyl oestradiol during most of the uterine life (GD 7–21), including the period shown by Rayner *et al.* to be the most sensitive period in utero for mammary glands (GD 17–19), and during their post-natal life from birth until necropsy PD 21–22 (Rayner *et al.*, 2005). As follows, offspring in the current study were exposed during all time points critical for pre-pubertal mammary gland development. An important developmental stage for the mammary glands is puberty, where the glands undergo extensive proliferation. Some studies have shown that male and female mammary glands may only show extensive changes to exposure after puberty, with no or barely prominent changes before puberty (Brown *et al.*, 1998; You *et al.*, 2002; Wang *et al.*, 2006). As a consequence, it is desirable to evaluate the status of the mammary glands post-puberty and compare with the results from PD21–22 to obtain an overview of the developmental effects of ethinyl oestradiol pre- and post-natally on the mammary glands before and after this developmental stage.

Latendresse *et al.* revealed histological changes seen as hyperplasia in mammary glands of male 90-day-old S–D rats chronically exposed to ethinyl oestradiol in low concentrations close to 0.2 μg/kg/day in the diet during pregnancy, lactation and adult life (Latendresse *et al.*, 2009). However, this was the only age and exposure group where hyperplasia was observed at this low exposure to ethinyl oestradiol. In 50 days old male rats, mammary hyperplasia was only seen at 5 μg/kg. Male rats exposed from conception to weaning also showed mammary gland hyperplasia on PND 50 at 5 μg/kg. Thus, perinatal exposure to ethinyl oestradiol does affect mammary glands, but a chronic exposure continuing in adult life may affect mammary glands at lower doses. In the present study, no statistically significant changes were seen in males at 5 and 15 μg/kg/day of ethinyl oestradiol. This may reflect the difference in strain, the differences in dosing by gavage compared with dosing in the diet leading to a different internal dose or it may reflect that effects are only seen at high doses at this age. Another possibility is that histological sections may be able to show further changes at lower concentrations of ethinyl oestradiol compared with whole mount evaluation.

In female whole mounts, the current study showed statistically significant changes in two morphological analyses at 15 μg/kg ethinyl oestradiol, but not at 50 μg/kg. This may be because of a large variation in the measurements or a smaller group size in the highest dose group compared with the group exposed to 15 μg/kg/day of ethinyl oestradiol for these endpoints (Table 1, Fig. 3). As some parameters were not possible to measure in all specimens, the group size varied between 4 and 7 litters for female rats in the highest dose-group and between 6 and 8 litters for the dose group exposed to 15 μg/kg/day. Another possibility is suggested by Vandenberg *et al.*, showing a non-monotonous dose–response curve for morphological analysis in whole mounts in female mice PND 35 mammary glands in response to increasing doses of 17β-oestradiol ranging from 0.25 to 50 μg/kg/day from PND 25–35, with a maximal effect at 5 μg/kg/day (Vandenberg *et al.*, 2006). It is

possible that the presence of endogenous oestrogen in female rats cause this non-monotonous dose–response in female rats and not in male rats. Ethinyl oestradiol may act as an oestrogenic agonist at low doses, but higher doses of ethinyl oestradiol may inhibit the actions of endogenous oestrogen by competitive binding to the oestrogen receptor, as discussed for EDCs by Meyers *et al.* (Myers *et al.*, 2009). In addition, Vandenberg *et al.* suggest that oestrogen can have proliferative as well as apoptotic effects in the mammary glands, and these opposing effects may lead to differences in response at low and high dose levels (Vandenberg *et al.*, 2006). However, the possibility of non-monotonous dose–response relationships for ethinyl oestradiol is yet to be verified.

To our knowledge, no studies have been published examining mammary whole mounts from rat pups exposed to ethinyl oestradiol, yet many other oestrogenic compounds have been studied in whole mounts of mammary glands at PD 22. The results from the present study confirm the changes found by Cotroneo *et al.* (2002) showing increased branching and alveolar structures PND 21 in female S–D rats exposed pre-pubertally to oestradiol benzoate (Cotroneo *et al.*, 2002).

Taken together, this study shows that perinatal exposure to strong oestrogen enhances male and female Wistar rat mammary gland development PD21–22. Moreover, the most sensitive endpoints at PD21–22 in Wistar rats after exposure to oestrogenic-acting chemicals in morphological analysis of mammary glands in whole mounts differ between male and female rats, except for the overall density. Hence, the overall density is a good representative for branching and budding for both genders. In female rats, the ductal outgrowth may be represented by the distance between fourth and fifth gland, or by the relative growth to the lymph node. The gland differentiation may be evaluated by counting the number of TEBs in zone C.

There is an evident interest in studying the development of mammary glands, and a validation process with more method development and extensive evaluation of the mammary gland samples is important to obtain data that facilitates further considerations on inclusion of male and female rat mammary gland investigation as an endpoint e.g. OECD test guidelines for Reproductive Toxicity. It is desirable to find the most sensitive endpoints to standardize the study of mammary gland whole mounts and recommendations for enhancing mammary gland evaluation in guideline toxicology studies have been proposed (Makris, 2010). Moreover, a review by Rudel *et al.* (2011) showed that early life environmental exposures can alter mammary gland development, disrupt lactation and increase susceptibility to breast cancer. They concluded that assessment of mammary gland development should be incorporated in chemical test guidelines and risk assessment (Rudel *et al.*, 2011). Specified outgrowth parameters and developed criteria for density scoring may be useful for development of guidance for evaluation of the rat mammary gland as an endocrine sensitive endpoint. We recommend including mammary gland development as a novel endpoint in OECD Test Guidelines including TG 443 Extended One-Generation Reproductive Toxicity Study. In our further studies, we plan to continue the method development in general and provide data on the relevance and sensitivity of mammary gland development as a novel endpoint in OECD test guidelines. Further studies in post-pubertal and adult Wistar rats and exposure to weak oestrogenic compounds can be used to further validate the mammary gland as an endpoint in reproductive toxicity studies, and to elucidate how early life environmental exposures may alter mammary gland development, disrupt lactation and alter susceptibility to breast cancer.

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Panel discussion

Toine Bovee (Wageringen, Netherlands):
You used ethinyl oestradiol (EE_2) as a gold standard for a compound with oestrogenic activity, but it is not a pure oestrogen receptor (ER) agonist because it is also an androgen receptor (AR) agonist and an AR antagonist. Its antiandrogenicity might be the cause of the effects seen on the male prostate gland. EE_2, therefore, is probably not a good reference compound for ER agonists. When pesticides are being characterized as oestrogenic or antiandrogenic, it is important to know their mode of action. Dienestrol is a synthetic oestrogen and is a pure ER agonist: this might be a better reference compound and it would be useful to compare outcomes caused by dienestrol with EE_2. EE_2 might be a good reference compound for pesticides which have both oestrogenic and antiandrogenic activities.

Julie Boberg (Søborg, Denmark):

Studying the effects of EE_2 is relevant to the other endocrine disrupters in the environment all of which have different types of effects, and it is difficult to discriminate between pure oestrogenic effects and pure antiandrogenic effects. We have ongoing studies assessing mixtures of several different environmental chemicals each characterized as putative oestrogens or putative antiandrogens, including known pesticides and phthalates. We are looking at the mammary effects of so-called oestrogens and so-called antiandrogens compared to controls.

Ana Soto (Boston, USA):

A ''round robin'' examination organized by Susan Fenton has shown quantitatively similar results in rats and mice when assessing endocrine effects on mammary glands. The mouse mammary gland is monoplanar, more sensitive and easier to work with. Why do you use rats rather than mice?

Julie Boberg:

The rat mammary gland is three dimensional which causes difficulties, and it may be easier to work with mice. However, male mice only have rudimentary breast tissue, and it has been shown that mammary glands of male rats are particularly sensitive to endocrine disrupting chemicals. Also, rats are often used for regulatory studies and it is essential to get information on the sensitivity to mammary gland changes in rat strains used for regulatory studies.

Ana Soto:

The presence of male breast tissue in mice is strain specific, and we have seen effects of bisphenol A (BPA) in male mammary glandular tissue in mice.

Anna-Maria Andersson (Copenhagen, Denmark):

You see gross morphological changes in the breasts of rats exposed to oestrogens during fetal life, and you alluded to the increased risk of breast cancer. Do you think that prenatal exposure might make the breast tissue more sensitive to exposure to carcinogens or hormonal changes in later life?

Julie Boberg:

There are many studies indicating that prenatal exposure to endocrine disrupters alters the susceptibility to carcinogenesis and tumour induction later in life. It is also possible that responses to hormonal challenges are influenced by early exposure to endocrine disrupters but only a few studies have been performed and more research is required.

Anna-Maria Andersson:

Rat and human breast tissues are similar in many ways but there is one important difference. Humans are the only species with breasts outside of the lactating period and develop breasts at puberty. The sensitivity of breast tissue outside of pregnancy and lactation might be specific to humans.

Julie Boberg:

Rats also develop breast tissue at puberty.

Shanna Swan (New York, USA):

You stated that pregnant women are not exposed to ethinyl oestradiol (EE_2) during pregnancy because they stop taking the oral contraceptive pill in order to become pregnant. But that is not entirely true because some breakthrough pregnancies occur when women are not taking the pill properly. They do not realize that they are pregnant until later in gestation when they are still taking the pill resulting in exposure of the human fetus to EE_2. A few years ago Fred vom Saal suggested studying this aspect of human prenatal exposure to oestrogens and it is a survey which must be performed because of the magnitude of the exposure. Although only a small percentage of pregnant mothers continue to take the pill, overall there are a substantial number of fetuses exposed, and the effects will be missed if we do not look for them.

PAPER 2

Karen Riiber Mandrup, Pernille Rosenskjold Jacobsen, Louise Krag Isling, Marta Axelstad, Karin Dreisig, Niels Hadrup, Anne Marie Vinggaard, Ulla Hass, Julie Boberg.

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

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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

Perinatal exposure to endocrine disrupting chemicals with estrogenic activity can adversely affect reproductive development, but few studies evaluating estrogen-sensitive endpoints have been performed in Wistar rats. Therefore, 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, urethral slit length and number of nipples was observed in female offspring. Prostate mRNA levels were affected prepubertally. For male and female mammary gland and prostate histology the expected differences were weak or absent, possibly due to termination of exposure at weaning.

In conclusion, ethinyl estradiol affected offspring before puberty and gave rise to persistant genital malformations following withdrawal of ethinyl estradiol exposure. Female external sexual characteristics and changes in prostates and male and female 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 Wistar 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]. In humans, ethinyl estradiol is used in contraceptive pills which contain 20-50µg ethinyl estradiol, corresponding to 0.2-0.7µg/kg bw/day, and thus the doses selected in the present study (5, 15 and 50 µg/kg) were approximately 10 to 100 times higher than exposure of women taking contraceptive pills. It may be noted that endocrine-disrupting effects of estrogenic compounds have been observed at lower doses in some studies, but based on the traditional expectations of increasing responses at increasing doses, the selected doses were relatively high in order to increase the possibility of detecting reproductive effects. Increasing knowledge on low-dose effects have made it clear that non-monotonous dose response curves can be seen for various endpoints [15;16], but this was not addressed by the current dose selection.

Previous studies examining the developmental effects of ethinyl estradiol as a model for estrogenic activity have mostly used Sprague-Dawley (SD) and Long-Evans (LE) rats, and strain differences have been reported for sensitivity to effects of estrogenic compounds [17-19]. The present study was conducted in outbred Wistar rats in order to investigate whether this rat strain is sensitive to a potent estrogen. The examination of endocrine sensitive endpoints in Wistar rats is particularly important in a regulatory perspective, as a large proportion of toxicological studies are performed in Wistar rats. 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 [20-22], whereas perinatal ethinyl estradiol exposure has not previously been shown to affect

AGD or nipple retention in males [11;23]. In female offspring, AGDs have been shown to be affected by ethinyl estradiol, although findings are not consistent among studies [24-29]. In the few studies investigating nipple retention in females, no significant effects of estrogenic compounds were seen [5;28].

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 perinatal exposure to estrogenic chemicals on male mammary glands, whereas a shift to a female-like morphology in male mammary glands has been shown after exposure to estrogenic compounds in adulthood [12;30-33]. Data on early mammary development were presented in Mandrup et al (2012) [34], 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.

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 [28;35-37], whereas exposure to estrogenic compounds seems to delay sexual maturation in SD and LE males [14;38-40]. Ethinyl estradiol, genistein and bisphenol A, have also been shown to cause more animals to cycle irregularly [27;41;42], and studies investigating the effects on external genital malformations in females have shown an increase in the urethral slit length in SD rats exposed to ethinyl estradiol [29;43]. 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 [44;45]. 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.

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 SD or LE rats, and to investigate further the influence of

ethinyl estradiol on AGD, nipple retention and changes in mammary glands. The study 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 GD21and from pup day (PD) 2 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). 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. From PD 23 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). 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.

All examined endpoints are listed in Table 1 by age and gender.

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 [47].

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), bulbourethral glands, and 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) [48] and in male offspring puberty onset was assessed as time of preputial separation (PPS) [40;49]. Females were examined daily from PD27 and males from PD34. The age and weight at VO or PPS were recorded.

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 [51]. 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) [52;53].

Necropsy PD50 and 55

On PD50 (females) or 55(males) offspring one male and one female per litter were 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.

Necropsy PD 90

On PD90 the remaining one female and one male per litter were anesthetised in CO_2/O_2 and decapitated. 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 from females: Liver, ovaries and uterus. The uterus was weighed when intact as well as after draining. From males, the following organs were excised and weighed: Liver, testis, epididymis, ventral prostate, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), and bulbourethral glands, 4^{th} mammary glands were excised from both males and females. Epididymis, ventral prostate, ovaries, uterus and 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) [54] 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) [55] 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) [56].
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). Female mammary glands were evaluated bearing the stage of estrous cycle in mind, as verified by stained smear and uterus histology. 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. 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, Estrogen 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 [57]. 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) [58]. 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 2). Additionally, at 50 µg/kg, the gestation length was increased ($p<0.001$) (Table 2). 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 2). Body weights of offspring at necropsy are shown in Table 3 and 4. 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 (data not shown). In females, 5 of 16 animals

(31%) 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 (11%), 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.

Organ weights

At PD21, the ventral prostate weights were significantly decreased in the group exposed to 15 µg/kg ($p<0.05$; Table 3). 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 3). No organs were weighed from males PD55. No statistically significant changes were found in organ weights of males PD90 (Table 4).

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 3) (data not shown for relative organ weights). On PD50 and 90, no changes in the weights of ovaries and uterus were found (Table 4). 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 5). 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 5). Reactive hyperplasia was observed in prostates from all males with moderately severe inflammation (Table 5).

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 SD and LE rats, and in the current study we examined effects in Wistar rats, which are commonly used in regulatory toxicology studies. The study included endpoints that have not previously been thoroughly investigatedand revealed 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 affected mainly mammary glands and prostate.

Effects on dams and pup growth

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]. Gestational length was increased in the highest dose-group, and to our knowledge this has not been reported for ethinyl estradiol before [27;28;43], but a prolonged gestational length has been reported for dietylstilbestrol (DES) [59;60].

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;29]. However, at weaning, this effect was smaller and only apparent in female offspring. This delayed growth of offspring may be a sign of toxicity, but could also 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 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 is in agreement with previous findings of increased AGD in female Long Evans rats at the same dose level and dosing period (GD7 to PND18) [28]. However, one study with similar exposure and dosing period found no effects of treatment in SD rats [29], and a multi-generation study in SD rats showed no changes in female AGD in F1 offspring at comparable doses [25;27]. It may be speculated that strain-specific differences are present with SD rats being less sensitive than LE or Wistar rats regarding estrogen-induced influences on female AGD.

There are only few reports describing effects on the number of nipples in females in the open literature, however, not with estrogenic exposure. In the National Toxicology Program (NTP) study hypertrophy of the nipples was found as an effect of ethinyl estradiol exposure [27], whereas Ryan et al (2010) did not observe increased number of nipples in female offspring in LE rats following ethinyl estradiol exposure at doses and dosing period comparable to the present study [28].

The increased urethral slit length at PD50 and PD90 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 [29;43]. Overall, ethinyl estradiol seemed to affect female external genital development. Changes in female external genitals were seen in neonates (increased AGD) as well as adults (increased urethral slit length), suggesting that effects on the genitals are persistant and present after withdrawal of the exposure.

Male and female mammary glands

In adult mammary glands, no statistically significant changes were found, but in adult female mammary glands, a trend to increased lobular development was observed, indicating advanced differentiation. Although this was not statistically significant

these trends may reflect persistent effects of ethinyl estradiol on mammary glands. As a small number of glands were evaluated the power to detect statistically significant changes on score measures is low, and it is considered important to pay attention to trends and findings that may suggest an effect of exposure, as stressed in OECD guidance documents for histological evaluation [61].

Changes in both female and male adult mammary glands were expected as lobular development (lobular hyperplasia) and increased secretory activity in females and feminization of male mammary glands have been described previously for SD rats [13;14;31]. 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 female SD rats exposed postnatally to genistein [14;62]. 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, as was expected based on the increased secretory dilation of alveoli seen in adult female SD rats after perinatal exposure to 17β-estradiol [31]. In that study, pups were exposed continuously through the diet until the day of necropsy, and as the same changes were found in adults exposed exclusively in adulthood [31], increased secretory activity may be expected only for adult estrogen exposure.

No clear indications of histological changes in male mammary glands were found, although epithelial hypertrophy was expected based on previous studies on estrogenic compounds. Our previous study on male SD rats exposed to a mixture of phytoestrogens showed a significantly higher incidence of hypertrophy in adulthood after exposure from GD 7 to PND 21 [57]. Another study on male SD rats exposed perinatally and after weaning to genistein showed increased alveolar hypertrophy and hyperplasia of male mammary glands PND50 [63]. In the current study, histological examination showed a tendency towards a higher prevalence of adult males with hypertrophic mammary epithelium, but this was not statistically significant, and hypertrophy was common in controls (50%).

No signs of feminization of the male mammary glands were present, as no increase in secretion or changes in tubuloalveolar pattern were observed. This is in accordance with the lack of feminization with tubuloalveolar growth in adult male mammary glands after dietary exposure of SD rats to 50 ppb ethinyl estradiol (approximately equal to 5 µg/kg) from conception until adulthood [12]. In contrast, adult exposure to high doses of estrogens (500 to 2500 µg/kg) has been shown to feminize mammary glands [31], and it has been hypothesized that feminization of the mammary gland is likely caused by hyperprolactinemia [64].

Although male mammary gland development was altered at PD 22 [34], persistent effects are likely more marked with continued exposure than perinatal exposure only. Further studies on cancer development in aging rats may determine whether these early changes in male mammary gland development can lead to persistent or delayed adverse effects.

Our previous study on prepubertal offspring from the same litters showed advanced development of mammary glands [34]. 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 [34]. 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 a low power for detection of persistent effects in adults due to the low number of animals examined.

Reproductive endpoints in female offspring

An earlier VO was expected in this study, as other studies have reported an earlier age of sexual maturation following perinatal exposure to ethinyl estradiol [27;28]. Although 11% and 31% of female offspring exposed to the two highest doses of ethinyl estradiol were in puberty at the first day of registration, effects were not statistically significant.. 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 [67]. 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). An increased incidence of abnormal estrous cyclicity was expected, as others have shown an increased length of cycles, an increased number of abnormal cycles or changes charachterized by persistent estrous in rats following exposure to ethinyl estradiol [27] [68] [25]. In the present study, no statistically significant effects on estrous cyclicity were observed, although irregularly cycling females were observed only in ethinyl estradiol exposed groups (7 of 26 exposed females). The lack of significant effects may be due the small group size and to the short dosing period terminating at weaning in contrast to the studies reported in the literature.

Reproductive endpoints 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 not at 50 µg/kg, and not in adulthood. Marked effects on adult male reproductive organ weights have been observed in other studies on perinatal exposure to comparable doses of 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 LE 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 seen in prepubertal but not in adult males. As no trends to decreasing adult male reproductive organ weights were seen in this study, it may be proposed that strain differences in sensitivity may explain these conflicting findings. Wistar rats have previously been found to be less sensitive than Fisher 344 rats to changes in male reproductive weights and histology [17], but no direct comparison with LE rats has been presented.

Histological changes in testes and epididymides were not observed but have been described in previous studies at higher dose levels or with adult exposure to ethinyl estradiol [69-71]. In a comparable study on subcutaneous exposure of SD rats to ethinyl estradiol in the neonatal period, only doses above 100 µg/kg induced histological changes in epididymis and testes, and the lack of histological changes in the current study were thus expected.

Prostate weight has previously been found to be affected by estrogenic compounds in a non-monotonous manner, but in those studies the opposite pattern was observed, i.e. increased prostate weight at low levels (0.015 µg/kg of estradiol benzoate) and decreased prostate weight at high doses (1.5 and 15 mg/kg bw) [18]. Comparable patterns have been reported in mice exposed to estrogenic compounds [72;73]. Our observation of reduced prostate weight at 15 µg/kg was thus expected, but the lack of effect at 50 µg/kg cannot be readily explained.

Estrogenic compounds may alter expression of ER responsive genes such as IGF-1 [74], Compl C3 or ODC. In addition, reduced ERα mRNA and protein levels have been found in adult rat prostates following perinatal soy exposure [75]. 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 [76], 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 [77]. Prostate weight was correlated to the PBPC3 mRNA level. Such a correlation has been described previously for flutamide [78]. 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 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 [44;45]. 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 SD 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 [79] non-monotonic dose-response patterns on prostate development have been described for natural and synthetic estrogens administered prenatally or neonatally to mice and rats [18;73].

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 perinatal exposure to ethinyl estradiol (27-63 µg/kg in the diet) [13;14] or other estrogenic compounds [8;38;80]. In a multi-generation study conducted by the NTP, delayed PPS was seen at the highest group of 50 ppb (approximately 4 ug/kg) but only in the F2 generation. In contrast, their range-finding study indicated accelerated PPS at 5 and 25 ppb (approximately 1.1 and 5.5 µg/kg , respectively) but not at higher doses [27]. The lack of effects on PPS in the current study may be due to the short dosing period compared to other studies showing delayed PPS following perinatal exposure to ethinyl estradiol [13;14] or other estrogenic compounds [8;38;80]..

*Refinement of reproductive toxicity studies*When planning animal studies, the number of animals should be appropriate, and too high or too low numbers of animals should be avoided. When evaluating studies in the published scientific literature it becomes clear that in many biological disciplines, a study of ten mated dams per group as in the current study may be considered a large study. This study (and a vast amount of evidence from toxicological studies) shows that for several reproductive endpoints including endpoints of a dichotomous nature, moderately sized studies are not sufficient to demonstrate presence or absence of effects [67]. 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 such endpoints, even in the present case of a potent estrogenic chemical.

Also selection of appropriate exposure periods and rat strains is necessary to ensure a high sensitivity of the selected endpoints. For some endpoints, studies with continued exposure to estrogenic chemicals appeared to show more marked effects than seen in 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 [81]. Most studies on e.g. mammary gland morphology and histology is, to our knowledge, studied in SD or LE 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. Further refinement of reproductive toxicity studies could be obtained by developing 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. Concomitantly, this would reduce the present need for a high number of experimental animals in reproductive toxicity 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, estrogen-regulated gene expression was increased dose-dependently and a decreased ventral prostate weight was seen at 15 µg/kg. Changes in mammary gland histology, timing of sexual maturation and estrous cyclicity were not statistically significant, but showed similar trends as observed in SD and LE 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. Additionally, the low power when dealing with data of a dichotomous nature should be should be carried in mind when evaluating or planning studies on possible endocrine disrupters.
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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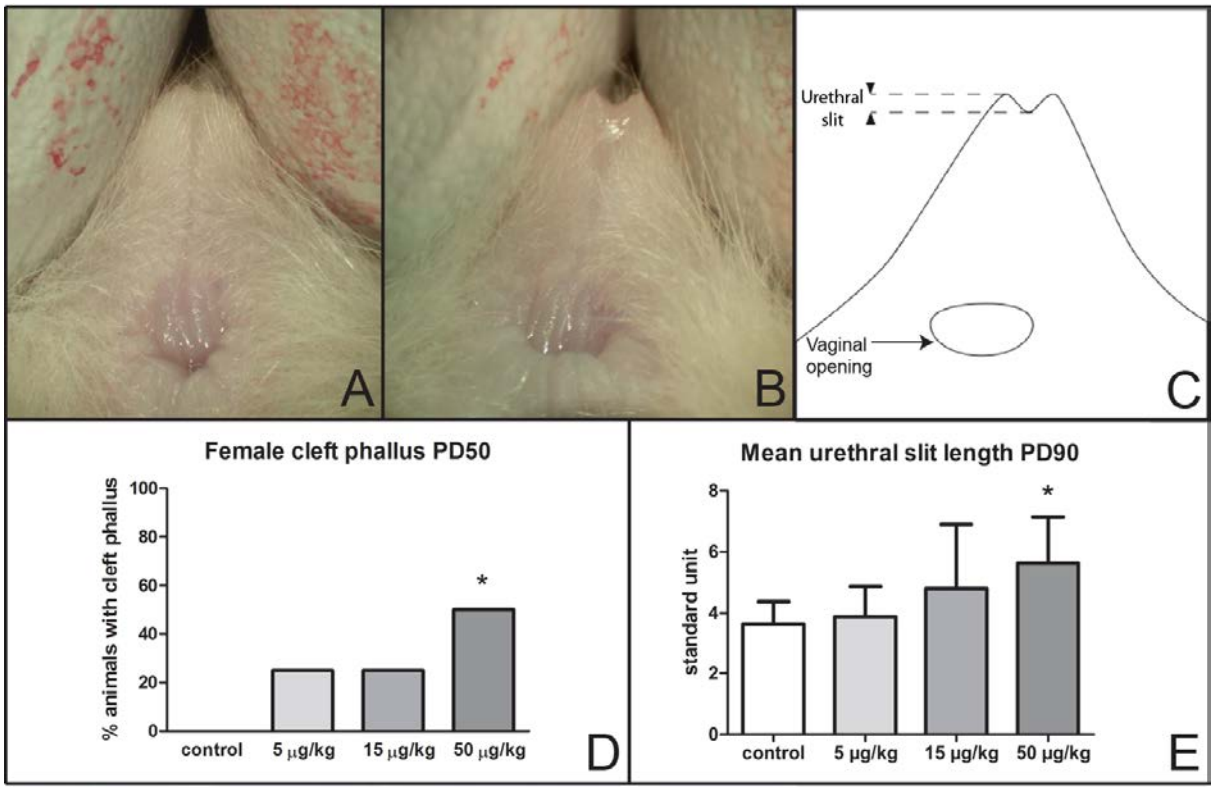

Figure 1. Female external genital malformations (cleft phallus). A, control animal with a shallow cleft. B, female from high dose group with a deep cleft/ large urethral slit length. C, schematic drawing of female B showing how the urethral slit length was measured. D, Prevalence of females with deep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

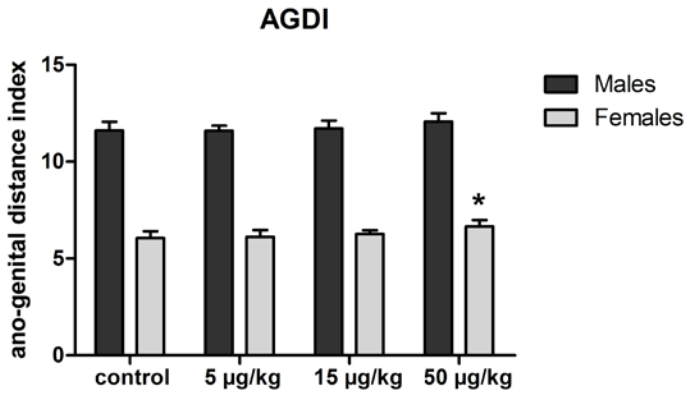

Figure 2. Ano-genital distance index (AGDI). Ano-genital distance adjusted for body weight for male and female offspring PD14.

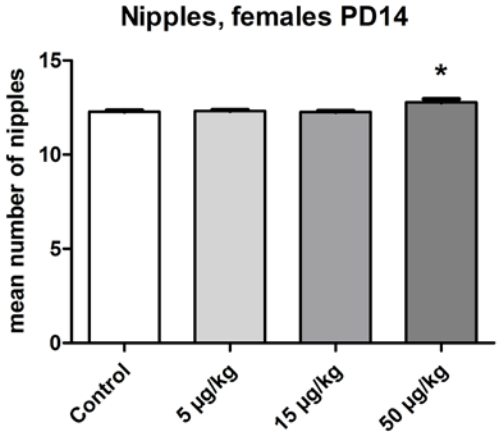

Figure 3. Number of nipples in female offspring PD14.

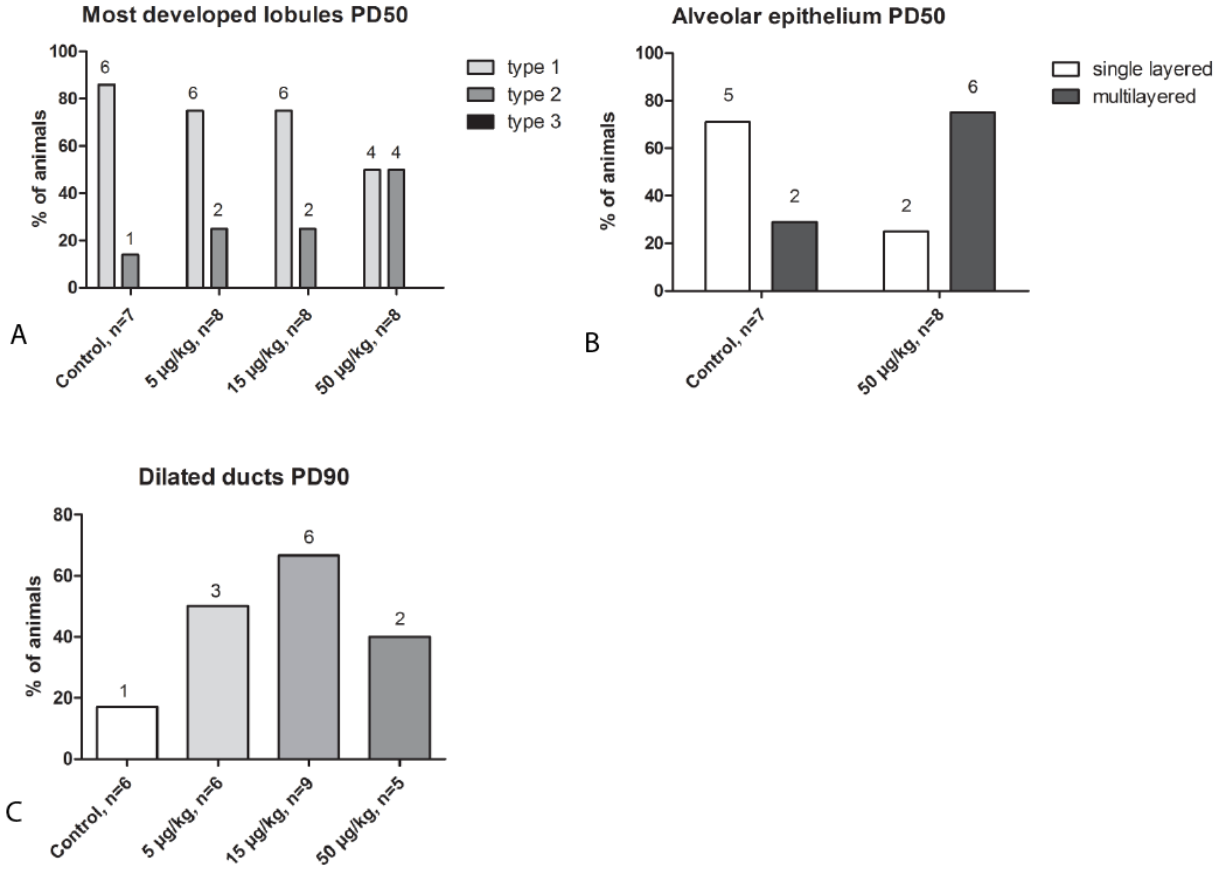

Figure 4: Adult female mammary glands. A, Most developed type of lobule in female whole mounts PD50. B, Histological evaluation of alveolar epithelium PD50. C, Histological evaluation of dilatation of mammary ducts PD90.

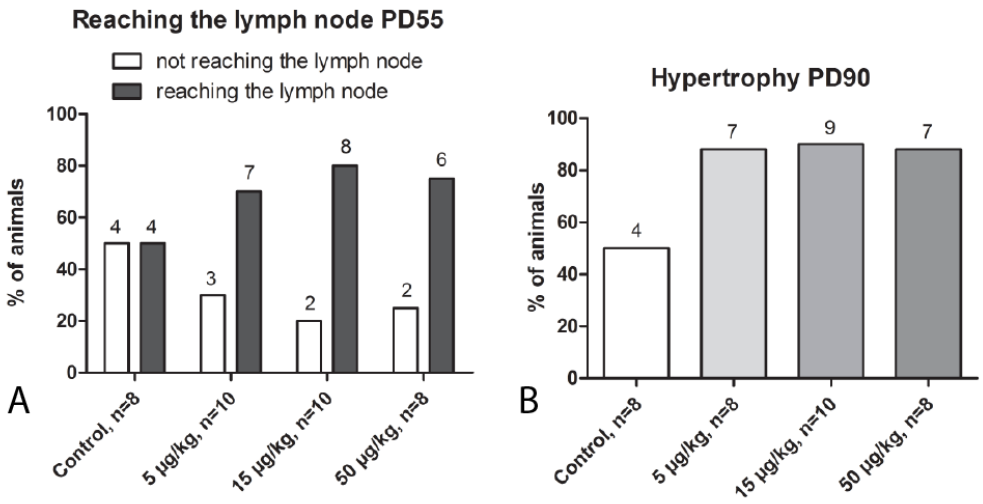

Figure 5: Adult male mammary glands. A, male mammary glands reaching the lymph node, evaluated in whole mounts PD55. B, Hypertrophy of mammary epithelium evaluated in histologic sections PD90.

Table 1 Examined endpoints

Age	Gender	Endpoint
PD 1	Male/female	AGD
PD 14	Male/female	Nipple retention
PD 21	Male	Organ weights (testis, ventral prostate, epididymis, seminal vesicle, LABC, bulbourethral gland, liver) Mammary gland whole mounts Thyroid histology Prostate gene expression (IGF-1, AR, TRPM-2, actin, Complement C3, PPAR α PPARγ, ER-α, ERβ, ODC, PBP C3, 18s, β-actin)
PD 22	Female	Organ weights (uterus, ovary, liver, thyroid) Mammary gland whole mounts
PD 27-41	Female	Puberty onset (vaginal opening)
PD 34-48	Male	Puberty onset (preputial separation)
PD 75-90	Female	Estrous cyclicity
PD 50	Female	Mammary gland whole mounts Mammary gland histology Organ weights (uterus, ovary)
PD55	Male	Mammary gland whole mounts Mammary gland histology
PD 90	Female	Organ weights (uterus, ovary, liver) Histology of mammary gland, uterus, ovary
PD 90	Male	Organ weights (testis, epididymis, ventral prostate, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands, 4^{th} mammary gland) Histology of mammary gland, ventral prostate, testis, epididymis

Table 2. 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*****
No. nipples males	0.05 ± 0.09	0.00	0,02 ± 0.06	0.02 ± 0.05
No. nipples females	12.28 ± 0.24	12.31 ± 0.16	12.27 ± 0.20	**12.78 ± 0.49****
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 3. 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 4. 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 5: Number and prevalence of males with a given histological findings in ventral prostate PD90

		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%)

PAPER 3

Julie Boberg, Karen Riiber Mandrup, Pernille Rosenskjold Jacobsen, Louise Krag Isling, Niels Hadrup, Line Berthelsen, Anders Elleby, Maria Kiersgaard, Anne Marie Vinggaard, Ulla Hass, Christina Nellemann.

Endocrine disrupting effects in rats perinatally exposed to a dietary relevant mixture of phytoestrogens.

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Title: Endocrine disrupting effects in rats perinatally exposed to a dietary relevant mixture of phytoestrogens

Running title: Endocrine disruption by phytoestrogen mixture

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Abstract

Dietary phytoestrogens may prevent certain human diseases, but endocrine activity has been reported in animal studies. Sprague-Dawley rats were exposed perinatally to a 1-, 10- or 100-fold "high human dietary intake" mixture of 12 phytoestrogens consisting of mainly the lignan secoisolarici resinol and the isoflavones genistein and daidzein.

This mixture induced persistent adverse effects, as male mammary glands showed hypertrophic growth. In young pups, no effects on reproductive organ weights were seen, but a reduced anogenital distance in newborn male pups exposed to the mixture indicated an anti-androgenic mode of action. Decreased serum estradiol was seen in genistein-exposed dams. This study indicated adverse effects at high intake levels in rats, but does not provide evidence for risk of phytoestrogen-mediated endocrine disruption at normal human dietary consumption levels. Further studies are warranted to increase the knowledge upon which risk assessment on dietary phytoestrogen exposure during pregnancy and infancy is based.

Highlights (3-5 bullets, optional)

- High doses of a dietary relevant mixture of phytoestrogens induced endocrine disruption in rats
- Mammary gland histology was altered in adult males after perinatal exposure
- Anti-androgenic effects on anogenital distance were present in young males
- An excessive human intake of phytoestrogens from dietary supplements or special dietary habits may contribute to endocrine disruption following exposure during fetal life

Keywords: mammary, histology, daidzein, genistein, secoisolarici resinol, lignans, diet, anti-androgenic

Abbreviations

AGD	Anogenital distance
DMBA	7,12-Dimethylbenz(a)anthracene
GD	Gestation day
MNU	*N*-Nitroso-*N*-methylurea
PND	Postnatal day
SECO	Secoisolariciresinol
TEB	Terminal end bud

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1. Introduction

Phytoestrogens, i.e. naturally occurring plant compounds with estrogenic actions, are believed to have chemopreventive effects on a variety of human diseases including cancers, through the ingestion of soy-based foods [1]. Concern has been raised, however, of potential adverse effects due to the estrogenic and other endocrine activities of these compounds.

Isoflavones such as daidzein and genistein are found in soy beans and other plant products, and humans are commonly exposed to these compounds through soy milk, tofu and other soy containing products [2]. Lignans including secoisolarici resinol (SECO) are found mainly in flax seeds, but also in sunflower, sesame, and pumpkin seeds, and a high intake is seen in populations with a high consumption of e.g. rye bread containing these seeds [3]. Rat studies on daidzein, genistein, SECO and SECO metabolites enterolactone and enterodiol have shown adverse developmental and reproductive effects in males and females [1;4-6]. Among these effects, changes in mammary gland morphology and tumor incidence have been reported in reproductive studies on SECO and genistein [1;7]. Postnatal exposure of rats to 10% dietary flaxseed or SECO diglucoside reduced incidence and numbers of tumors induced by 7,12-Dimethylbenz(a)anthracene (DMBA) [8]. Other studies have shown that exposure to flax seed containing SECO during early development enhances mammary growth [9] and increases the susceptibility to carcinogen-induced mammary tumors [10], and it appears that the possible protective effects of these compounds are highly dependent on the time window of exposure.

For genistein, developmental and reproductive toxicity in rats is known, as evidenced by transient decreased offspring body weight at doses from 7 mg/kg bw/day, decreased anogenital distance in male and female pups, reduced litter size, reduced pregnancy rate, altered estrous cyclicity, altered ovarian and prostate histology and accelerated vaginal opening at 35 to 44 mg/kg bw/day [5]. A National Toxicology Program expert panel considered adverse reproductive or developmental

effects unlikely for human adults with exposures up to 0.43 mg/kg bw/day, and negligible concern for neonates and infants consuming 0.01-0.08 mg/kg bw/day of genistein in soy formula. However, the combined exposure to endocrine active compounds in human diet needs to be considered to determine whether human phytoestrogen intake may have harmful effects. In the current study, we examined the endocrine disrupting effects in rats of a mixture of phytoestrogens. The mixture composition was based on the composition of phytoestrogens in human diet in order to address the shortcoming controversy of doing risk assessment based on single compound exposure, whereas real-life human exposure is to a mixture of compounds with different endocrine effects. The mixture composition is listed in Table 1 and based on the upper range of exposures determined by Hedelin et al., 2006 [11]. This mixture was administered by gavage in order to control the exact exposure in doses that were 1-fold, 10-fold or 100-fold of "high human dietary intake". As indicated in Table 1, the main components of the mixture are secoisolarici resinol, genistein and daidzein with a smaller content of other resinols and a very low content of isoflavonoids, SECO metabolites and coumestrol. Hedelin et al. calculated daily phytoestrogen exposure levels for humans based on a food frequency questionnaire distributed to Swedish prostate cancer cases and corresponding controls (mean age 68 years), combined with analytical data on content of phytoestrogens in food products [11]. These data were used as surrogate data for "high human dietary intake" of pregnant women, although there may be differences in diets of elderly men and young pregnant women. To control background exposure to phytoestrogens, a soy-free rat chow was used as a background diet. This diet as well as the ambient environment may contain other estrogenic compounds, and this study was thus performed with a background exposure to estrogenic compounds, as also experienced by humans.

In vitro studies on the 12 compounds carried out in our lab showed that genistein inhibited testosterone production and that genistein, daidzein and a mixture of lignans including SECO could

increase estradiol production in a human adrenal steroid producing cell line [12]. None of the phytoestrogen mixtures tested acted as antagonists of the androgen receptor in vitro, but a mixture of lignans was found to be an aromatase inducer [12]. Another study in adrenal cells has shown a general inhibitory effect of lignan metabolites on steroid synthesis in adrenal cells, an effect that could in turn contribute to an anti-androgenic mode of action [13]. In vivo studies on SECO or flax seed containing SECO have indicated adverse reproductive effects, as altered anogenital distance, altered estrus cyclicity and altered ventral prostate weights were observed in rats exposed to flaxseed in the diet [6;14-16]. Decreased male and female AGD at PND 3, altered estrus cyclicity, and increased uterus (prepubertal), ovary (adult) and prostate (adult) weights were observed in rats exposed in utero and via lactation to 10% flax seed corresponding to 12 mg/kg bw/day of SECO [16]. In our study, the middle and high dose included 17.4 mg/kg bw/day and 174 mg/kg bw/day of SECO, respectively, i.e. higher doses than the effective doses in the study on dietary flax seed exposure.

The genistein dose (19.4 mg/kg bw/day) contained in the highest mixture dose was lower than those being effective in studies on perinatal exposure (35-44 mg/kg bw/day) [5]. It is thus expected that SECO contributes markedly to the possible reproductive effects of the current phytoestrogen mixture.

Daidzein is metabolized to equol, which has been shown to have anti-androgenic effects in adult rats by reducing prostate and epididymal weights through binding of dihydrotestosterone and thereby preventing it from activating the androgen receptor [4]. Formononetin and biochanin A were potent inhibitors of testosterone production and inducers of estradiol production in vitro [12], and the weak estrogen receptor agonist coumestrol is known to reduce sex hormone levels in young male rats at doses far above those applied in the current study [17]. Little is known about the effects of resinols other than SECO on estrogenic or anti-androgenic endpoints.

This paper describes effects of a human relevant mixture of phytoestrogens on hormone sensitive endpoints including anogenital distance (AGD), nipple retention, mammary development and chemically induced mammary tumor incidence in rat offspring exposed during pregnancy and lactation.

2. Materials & Methods

2.1 Test compounds

Genistein (CAS 446-72-0), daidzein (CAS 486-66-8), equol (CAS 66036-38-2), formononetin (CAS 485-72-3), biochanin A (CAS 491-80-5), enterodiol (CAS 80226-00-2), and coumestrol (CAS 479-13-0) were all purchased from Sigma-Aldrich (Milwaukee, WI, USA). Enterolactone (CAS 78473-71-9), secoisolarici resinol (CAS 29288-59-8), matairesinol (CAS 580-72-3), lariciresinol (CAS 27003-73-2), and pinoresinol (CAS 487-36-5) were purchased from Arbo-Nova (Turku, Finland). N-methyl-N-nitrosourea (MNU, CAS 684-93-5) was purchased from Sigma-Aldrich (Milwaukee, WI, USA).

2.2 Animals and dosing

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 of the National Food Institute at the Technical University of Denmark. 80 time-mated Sprague-Dawley rats (Taconic M&B, Denmark, bodyweight approx. 230 g) were supplied at day 3 of pregnancy. The study was performed in 3 blocks separated by one week and with equal distribution of dose groups. The dams were randomized into 5 groups of 16 with similar body weight distributions and housed in pairs under standard conditions. Semi-transparent plastic cages with Tapvei aspen bedding were situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light

starting at 9 p.m., light intensity 500 lux, temperature 21 ± 2°C, humidity 50% ± 5%, ventilation 8 air changes per h). Food (Soy free Altromin 1314) and tap water were provided *ad libitum*. Dams were dosed daily by gavage from gestation day (GD) 7 to postnatal day (PND) 13 with either vehicle (corn oil), a mixture of 12 phytoestrogens (both lignans and isoflavonoids) or genistein alone. Doses were selected from "high human dietary intake" determined by Hedelin et al., 2006, in a Swedish population (Table 1). Control group: corn oil only. "Mix 2" group: 1 x "high human dietary intake" phytoestrogen mix (total of 2.13 mg/kg bw/day). "Mix 21" group: 10 x "high human dietary intake" phytoestrogen mix (total of 21.3 mg/kg bw/day). "Mix 213" group: 100 x "high human dietary intake" phytoestrogen mix (total of 213 mg/kg bw/day). "Genistein 213" group: genistein 213 mg/kg bw/day.

Anogenital distance (AGD) was measured in male and female pups on the day of birth using a dissecting scope with an ocular reticle. AGDi was calculated as the AGD divided by the third root of birth weight. At PND 13 all pups were weighed and examined for the presence of nipples/areolas, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. The nipples were counted and no distinction was made between the retention of an areola or a nipple. However, a technical error was identified, as areolas (dark coloration of skin) were only assessed in the last of the 3 blocks. The number of nipples in female rats is normally 12-13 versus zero in males. AGD and nipple retention was recorded by the same technician skilled in these measurements and blinded to exposure group.

On PND 13, one male and one female pup per litter were randomly selected for autopsy and mammary analyses. On PND 22, one female pup per litter were randomly selected for autopsy and mammary analyses, and one male per litter and two females per litter were weaned and kept for examinations in adulthood. Remaining pups were sacrificed and blood was collected for hormone analyses. Weaned rats were caged in pairs of same sex and exposure. Age at sexual maturation was

investigated in all weaned rats by recording the day of vaginal opening in the females and cleavage of the balano-preputial skinfold in the males. The latter was done by observing when the prepuce, which is fused to the glans penis until the onset of puberty, could be fully retracted. The day sexual maturation was observed, the weight of the animals was registered.

At PND 50, one randomly selected female per litter (MNU-females) was exposed to 50 mg/kg bw of *N*-Nitroso-*N*-methylurea (MNU) in a 2% solution in isotonic saline as an intraperitoneal injection of a total amount of 2.5 ml per kg bw. MNU acts as a mammary tumor inducer in rats [18]. Rats were palpated for tumors twice a week from PND 64 onwards, and age of mammary tumor detection was noted.

2.3 Autopsies

On PND 13 one male and one female per litter were sacrificed by decapitation and blood was collected for hormone analyses. From females the 4th mammary glands were excised for whole mount preparation and gene expression, respectively. From males, the 4th mammary glands were excised for whole mount preparation. Ventral prostate was weighed and placed in RNAlater (Life Technologies Europe BV, Naerum, Denmark) for gene expression analysis. Testes were weighed and one testis was placed in Bouin's fixative for later histological examination, and the other was placed in an empty tube and snap-frozen in liquid nitrogen for measurement of testicular testosterone content. Livers from males and females were weighed and placed in RNAlater for gene expression analysis.

On PND 22, one female per litter was sacrificed by decapitation after CO2/O2 anesthesia and blood was collected for hormone analysis. The 4th mammary glands were excised for whole mount preparation and gene expression, respectively, and the uterus and one ovary were weighed and fixed in formalin for histological examination.

Adult males were sacrificed by decapitation after CO2/O2 anesthesia at PND 150 and the 4th mammary glands were excised and fixed in formalin for histological examination and whole mount, respectively. One testis and the ventral prostate were weighed and placed in RNAlater for gene expression analysis. The other testis was fixed in Bouin's fixative for histological examination.
Females exposed to MNU on PND 50 (one per litter) were sacrificed by decapitation after CO2/O2 anesthesia when tumor size approximated 20 mm in accordance with in-house ethical guidelines. Rats were examined, tumor number and sizes were recorded, and tumors were excised and fixed in formalin for histological examination. When mammary tumors were detected in 50% of controls, the remaining MNU-females were sacrificed (PND 132).
Females not exposed to MNU were sacrificed by decapitation after CO2/O2 anesthesia at PND 135-150 in CO_2/O_2 anaesthesia, blood was collected for hormone analysis, and a vaginal smear was collected for determination of estrous cycle stage and stained by the adapted Papanicolau procedure [19] (Gill's hematoxylin, Orange G6 and eosin-azure 50, provided by VWR, Gentofte, Denmark). It was the aim to sacrifice animals in proestrous, which was determined by measurement of the electrical impedance of the epithelial cell layer of vaginal mucosa by inserting a Vaginal Impedance Checker (VIC, Model MK-10C, Muromachi, Japan) into the vagina. If the measured value was 3 kΩ of impedance or above, the animal was considered to be in proestrous. As this measurement was somewhat variable, the accurate stage of estrous cycle was determined by evaluation of vaginal smears together with histological evaluation of uterus and ovary. A few animals were in anestrous and were omitted from the histological evaluation of mammary glands, ovaries and uteri, and evaluations of the remaining animals were performed bearing the stage of the estrous cycle in mind.
From each female one of the 4th mammary glands was excised for whole mount preparation and the 2nd mammary gland, uterus and one ovary were excised, weighed and fixed in formalin for

histological examination. The other ovary was weighed and placed in RNAlater for gene expression analysis.

2.4 Histology

Tissue samples for histological examination were routinely processed, embedded in paraffin, and sectioned. All sections were evaluated blinded to treatment groups in adult males and females. Hematoxylin and eosin (HE) stained sections from adult female mammary glands were evaluated for vacuolisation and lobuloalveolar pattern defined as mammary epithelium forming lobular units with no alveolar structures (no lumen, no one-layered alveolar epithelium, e.g. loss of female alveolar architecture). HE stained sections from adult male mammary glands were evaluated for hypertrophy and vacuolisation of mammary epithelium and secretory material in alveoles and ducts. Additionally, adult male mammary glands were stained with periodic acid Schiff (PAS) and mucicarmine. PAS stained sections were evaluated for PAS-positive secretion. Mucicarmine stained sections were evaluated for mucin-positive secretion.

One HE stained section of each tumor were examined and classified according to the dominating histopathological type [20]. A semi-quantitative analysis was made for stromal (desmoplastic) reaction: No stromal reaction (0), mild stromal reaction (1), moderate stromal reaction (2), severe stromal reaction (3), and for stromal inflammation (infiltration with inflammatory cells in the stroma): No infiltration (0), mild = focal or few multifocal areas with low cell density (1), moderate = multifocal areas with low to moderate cell density (2), severe = multifocal areas with high cell density (3).

One HE stained section of each testis (PND 13 and adult) were evaluated histologically. Prepubertal testes were examined for lumen formation and developmental stage. Adult testes were examined for

degenerative changes and abnormal development. Apoptosis assessment by TUNEL staining of testes was performed in one section per testis as described in [21].

In one HE stained section of PND 22 ovaries, the number of multioocyte follicles per section was determined. From adult females, one HE stained section of the uterus and one HE stained section of the ovary from all rats were examined histologically. The accurate stage of the estrous cycle was determined by evaluation of uterine and ovarian histology as well as vaginal smear evaluation. Lumen size, epithelial folding and epithelial height were evaluated in uterine sections. The number of corpus lutei, nonatretic and atretic antral follicles, follicular cysts and granulosa cell hyperplasia (none, few nests, several nests and multiple nests/diffuse scored as 0,1,2,3 respectively) were evaluated in ovarian sections.

2.5 Whole mounts

Mammary glands for whole mount preparation were excised with the adjacent lymph node and spread on a glass slide. The tissue was covered with a coverslip and compressed for 1-2 hours before fixation in formalin overnight. Further processing was performed as described by Mandrup et al., 2012 [22].

Whole mounts were evaluated by an examiner blinded to treatment groups. Mammary development was examined by evaluating mammary whole mounts from 13 and 22-day old rats using a 1-4 score for density of the tissue and by measurement of the longitudinal and transversal expansion and the area of the mammary tissue. Whole mounts of adult males and females were evaluated for mammary development using a 1-5 density score. Density scores were given according to the extension of branching and budding of the gland, adjusted for age and gender. Moreover, the number of terminal end buds (TEBs) were counted in male and female mammary glands at PND 13

and were defined as tear-drop shaped structures with a diameter larger than 100μm as described by [23]. It was not possible to evaluate all parameters in some whole mounts due to missing parts.

2.6 Hormone levels

Trunk blood was centrifuged at 4000 g for 10 min at 4˚C, and kept at -80ºC until analysis of testosterone concentration. Steroids were extracted from plasma by solid-phase extraction using IST Isolute C18 SPE columns of 100 mg. The testosterone concentration was measured with a Delfia time-resolved fluoroimmunoassay (PerkinElmer, Wallac Oy, Turku, Finland). The detection limit of the assay is 0.3 nM and intra-assay variability is reported to 5.5-6% by the supplier. Values below the limit of detection were set to the same value as the detection limit.

2.7 Gene expression

mRNA was purified from RNAlater stored samples by use of the RNAeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesised by use of the Omniscript RT kit (Life Technologies Europe BV, Naerum, Denmark). Both procedures were done according to the descriptions by the manufacturers. mRNA levels were evaluated by quantitative (q)PCR using TAQ man probes in combination with specific primer pairs (Sequences are given in Supplementary Materials, Table S1). These were mixed withTaq Man Fast Universal PCR Master Mix (Life Technologies Europe BV, Naerum, Denmark) and then run on a Taqman 7900 HT qPCR apparatus (Applied Biosystems). Two methods were used to evaluate the results. Quantitation by use of standard curves as described previously [24] using beta actin values for normalization was used for the following genes: Insulin-like growth factor-1 (IGF-1), androgen receptor (AR), Transient receptor potential cation channel, Subfamily M, member 2 (TRPM-2) and Prostate specific binding protein polypeptide C3 (PBP C3) in the ventral prostate of PND 13 males, and PBP C3 and IGF-1 in

ventral prostate of adult males. Quantification by the comparative threshold cycle (C_T) method (without a standard curve) was employed for of the following genes: IGF-1, Estrogen receptor-α (ERα) and ERβ in female mammary glands PND 13 and PND 22, ERα in the ventral prostate of PND 13 males, ERα and AR in ventral prostate of adult males; AR and ERα in testes of adult males. 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). The 2-dCt values were then used for statistical analysis to test for differences between treatment groups.

2.8 Statistics

Non-processed, logarithmically transformed and square root transformed data were examined for normal distribution and homogeneity of variance. Normally distributed data were analyzed by ANOVA using single animal data. For datasets including more than one animal per litter, the litter was the statistical unit and included in the analysis of variance as an independent, random factor. AGD and AGDi data were analyzed for males and females separately, and with bodyweight as a covariate. Additionally, AGD and AGDi data from the genistein-exposed group were compared to controls by comparing differences of LS means. Organ weights and outgrowth measures on mammary whole mounts were analyzed using body weight as a covariate. Data that could not obtain variance homogeneity or normal distribution with transformation were examined in a non-parametric Kruskall-Wallis test followed by Dunn's test for multiple comparisons. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$; ***:$p<0.001$.

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 nipple retention, correlation structures between number of nipple/areolas and litter were modeled by the Generalized Estimating Equations method[25]. All statistical analyses were performed using the SAS procedure PROC GENMOD (SAS Institute Inc, Cary, NC, USA). SAS statistical software was used for the analyses (SAS Institute Inc, Cary, NC, USA).

Histological data were analyzed by Fisher exact test using SAS Enterprise 9.1. Exposed groups were only compared with controls separately in cases with overall statistically significant results. Data on tumor incidence were analyzed by Peto test (proc lifetest) for analysis of differences between groups in age of occurrence of first tumor and by Fisher exact test for comparison of tumor numbers at a fixed age at which more than 50% of controls had palpable tumors (PND 132). Mammary histology was analysed in GraphPad Prism 5 with a Fisher exact test.

Regarding gene expression, dC_T values were tested for normal distribution by the D'Agostino and Pearson omnibus normality test. Normal distributed data were analyzed by one-way ANOVA with post-test for linear slope or with Dunnet's test for control vs. phyto groups, as well as a t-test of control vs. genistein. Data that were not normal distributed were analyzed using a Kruskall-Wallis test followed by Dunn's test for multiple comparisons for control *vs.* mixture groups, as well as a Mann-Whitney test of control *vs.* Genistein.

3. Results

3.1 Perinatal development

AGD was assessed in offspring to evaluate anti-androgenic effects of the phytoestrogen mixture. A dose-related trend to decreasing AGDs at birth was seen in males exposed to the mixture, and significantly reduced AGD and AGDi at birth were seen in the highest mixture group compared to controls (Fig. 1A). For genistein exposed animals, AGD and AGDi were not significantly different from controls in Dunnett's post hoc test ($p=0.11$ and $p=0.13$). A high outlier from an abnormal litter with only one male and one female pup likely influenced the lack of statistical significance for the genistein group. Reductions in AGD and AGDi were statistically significant in genistein exposed male pups when comparing differences of LS means, i.e. a pairwise comparison of genistein and controls ($p=0.03$ and $P=0.04$, respectively). No changes in AGD were seen for females.

Data on nipple/areola retention were omitted due to an identified technical error, as areolas (dark coloration of skin) were only assessed in the last of the 3 blocks.

No changes in maternal weight gain, gestation length, post-implantation or perinatal loss, postnatal deaths, sex ratio, birth weights or body weights of offspring were observed (Table 2). No alterations of puberty onset were observed (data not shown).

3.2 Hormone levels and organ weights

Organ weights were unaffected by treatment at PND 13 (ventral prostate, testis, liver) or 22 (ovary, uterus) or in adulthood (ventral prostate, testis, ovary, uterus) (Table 3).

Reduced serum estradiol levels were seen in dams from the genistein group compared to controls, whereas the phytoestrogen mixture did not affect estradiol levels (Fig. 2A). A trend towards reduced plasma estradiol levels in female offspring was not statistically significant (Fig. 2B).

Males exposed to genistein had a statistically significant reduction in serum testosterone level compared to controls at PND 13 (Fig. 2C). The analyzed samples were pools of serum from 2-4 pups from the same litter, and the observed reduction was due to 3 out of 5 pooled serum samples from genistein exposed males having testosterone levels below the limit of detection, whereas all control samples had testosterone levels above the detection limit. At PND 22 (Fig. 2D) and in adulthood no statistically significant effect on testosterone was found. Testicular testosterone concentration was not changed by the phytoestrogen administration (Table 3).

3.3 Mammary whole mounts

Mammary development was evaluated in whole mounts of the 4th mammary gland of offspring at PND 13 (females and males) and PND 22 (females). No statistically significant differences between groups were observed for the area (Supplementary materials, Table S2), the longitudinal- or the transverse growth of the breast tissue at PND 13 or PND 22 (data not shown). A statistically significant reduction in density scores was observed at PND 13 in females from the lowest mixture dose group compared to controls, but no statistically significant differences in density scores were seen for PND 13 males or PND 22 females (Supplementary materials, Table S2). When including body weight as a covariate in the analysis of density scores of PND 13 females, there were no statistically significant differences between groups. No statistically significant differences between groups were found in the number of TEBs. In adult females and males no statistically significant differences in density score were seen between the groups. Areas with female-like branches were observed sporadically in male mammary glands PND 150 in all groups.

3.4 Mammary histology

Histopathology of mammary glands was evaluated in females PND 135 and males PND 150. In males, a dose-related increase in hypertrophy was observed and was statistically significant at the highest phytoestrogen mixture dose. An elevated number of males with hypertrophy was also seen in the genistein group, but this was not statistically significant. Among the 40 male mammary glands with hypertrophic epithelium, 11 were found to show a similar morphology to the apocrine changes seen in some human breast cancers [26] (Fig. 3C and 3F). These apocrine-like changes were defined as cells with abundant eosinophilic cytoplasm containing small eosinophilic granules and round to oval lightly stained nuclei as described by Durham and Fechner, 2000 [27]. Additionally, vacuolated mammary epithelium and secretory material in alveoli was observed more frequently in animals exposed to phytoestrogens than in controls, but this was not statistically significant (Fisher exact test for secretory material in alveoli in high dose mixture group: $p=0.06$) (Fig. 3D). In females, histological examination was performed on cycling animals. Four animals in the phytoestrogen mixture groups were in anoestrous and were not included for statistical analysis nor shown in Fig. 4. In females exposed to the phytoestrogen mixture, an increase in the prevalence of animals with lobuloalveolar pattern was seen, yet this was not statistically significant (Fig. 4B and 4C). No increase in vacuolization of the epithelium was observed with dosing. Vacuolated epithelium was not specific for a certain stage of estrous cycle, and the lobuloalveolar pattern observed was distinguishable from the typical lobules with tubuloalveolar pattern of animals in diestrous and metestrous [28]. PAS and mucicarmine stained male mammary glands showed no differences between the groups for PAS-positive and mucin-positive secretion.

3.5 Mammary tumors

Tumor latency after MNU exposure was not different between dose groups (Fig. 5). Animals were sacrificed at different ages for ethical reasons. Due to these age differences, tumor multiplicity data

were not considered relevant for statistical examination, but a qualitative data examination was performed and showed no apparent differences between groups. Histological examination of tumors showed that all tumors were malignant adenocarcinomas of variable size. A cribriform pattern was dominating at all doses, while in some exposed animals the papillary and comedo patterns were the dominating histopathological types. These differences between groups in dominating histopathological types were not statistically significant (Supplementary materials, Table S2). No differences between groups were observed with respect to stromal reactions or inflammation.

3.6 Histology of other organs

No group differences in testis histology were apparent at PND 13 and in adulthood. Apoptosis assessment by TUNEL staining of adult testes showed no treatment-related effects (data not shown).

Staging of vaginal smears, uteri and ovaries showed that not all animals were in proestrous (P) as intended, but several animals had entered estrous (E) and a smaller number of animals were in diestrous (D) or metestrous (M). All groups except controls had 1- 3 animals evaluated as being in anestrous as listed below:

Controls: 10 animals in P or E, 4 animals in D or M, 0 animals in anestrous. Mix 2: 12 animals in P or E, 3 animals in D or M, 1 animal in anestrous.Mix 21: 10 animals in P or E, 1 animal in D or M, 3 animals in anestrous. Mix 213: 14 animals in P or E, 1 animal in D or M, 1 animal in anestrous. Geni 213: 9 animals in P or E, 2 animals in D or M, 3 animals in anestrous.

Although anestrous appeared more frequently among exposed animals than controls, this was not statistically significant and may be a sign of pseudopregnancy, which could be induced by the use of vaginal impedance measurement.

Histological examination of uterus and ovary showed no dose-related changes.

3.7 Gene expression

Gene expression in female mammary glands showed no statistically significant changes in the expression of mRNA for IGF-1, ERα or ERβ on PND 13 or 22 (data not shown). Gene expression analysis of PND 13 ventral prostates showed no statistically significant differences between controls and exposed groups in the expression of IGF-1, AR, PBP C3, TRPM-2 or ERα (data not shown). In adult ventral prostate, ERα expression was very low and no differences in IGF-1, PBPC3, AR or ERα expression were observed (data not shown). In adult testes, no changes in AR or ERα mRNA expression were observed (data not shown).

4. Discussion (1460 words excl references)

In contrast to previous studies on SECO and genistein, the current study showed only few adverse effects on developing and adult male rats. Changes in female mammary glands and adverse changes in the male reproductive system were expected based on studies on genistein or SECO reported in the literature, but were not detected in the current study. However, this is the first study to describe reduced AGD in male pups and persistent adverse changes in male mammary glands following perinatal exposure to a phytoestrogen mixture containing mainly SECO and genistein.

4.1 Early anti-androgenic effects

Perinatal exposure to this phytoestrogen mixture induced anti-androgenic effects in young male rats, as a dose-related trend to decreased AGD was observed reaching statistical significance at the highest doseThe effect of the mixture was likely caused by the combined exposure to SECO, genistein and daidzein, as these were the main constituents of the mixture, and these compounds have all been associated with anti-androgenic effects [1;5;6]. Multiple modes of actions are known for the main constituent of the mixture, SECO, which can act as an estrogen, a steroid synthesis inhibitor, and an aromatase inducer and/or inhibitor [12;13].

4.2 Mammary gland effects

The phytoestrogen mixture affected male mammary glands, as an increase in the number of males with hypertrophic mammary epithelium was seen at the high mixture dose. Likewise, Delclos et al., 2001, observed hypertrophy of the alveolar and ductal epithelium in male mammary glands PND 50 after perinatal dietary exposure to genistein (from 25 ppm (approximately 2.5 mg/kg bw/day) and above) [29]. Comparable effects were seen in the animals exposed to genistein alone, but

unexpectedly, these were not statistically significant despite a high dose level of 213 mg/kg bw/day. One difference between these studies is dietary exposure versus exposure by gavage, but the apparently higher sensitivity in the dietary study may also reflect that exposure of offspring was continued until necropsy on PND 50 in that study.

Among the animals with hypertrophic epithelium in the current study, several were found to be morphologically similar to the apocrine metaplasia seen in some human breast cancers [26]. To our knowledge, apocrine changes have not been previously described in rats, and the human relevance of these apocrine-like changes in rats may be of interest for further investigation.

Moreover, presence of secretory material in alveoli of adult male mammary glands may be a sign of feminization and was observed more frequently in males exposed to phytoestrogens than in controls, but this was not statistically significant. As the power to detect statistically significant changes on binary measures is low, it is important to pay attention to trends and findings that, although not statistically significant, may suggest an effect of exposure to the test material, as stressed in OECD guidance documents for histological evaluation [30]. Similar effects were observed in a study with perinatal exposure to a mixture of pesticides with anti-androgenic properties [31], suggesting a feminization of the male mammary gland under the action of pesticides with anti-androgenic activity. Androgens are known to be responsible for masculinization of the mammary gland during pre- and postnatal development [32;33], but there are currently few studies on the actions of anti-androgenic compounds on the male mammary gland. Male rats appear to be particularly sensitive to endocrine disrupting compounds in this and other studies [7;29], and evaluation of male mammary gland histology is included in the recent OECD guideline for the extended one-generation study (OECD 443) [34].

In adult female mammary glands, a trend to increasing frequencies of lobuloalveolar pattern was seen in mixture groups indicating a possible masculinization of the mammary gland. In a comparable study using perinatal dietary exposure to genistein, histopathology of female mammary glands PND 50 showed hyperplasia with proliferation of alveolar complexes into compact lobules, which was statistically significant from 250 ppm (approximately 25 mg/kg/day) following pre- and postnatal exposure until PND 50 [29]. In the current study, the highest mixture dose contained 19.4 mg/kg bw/day of genistein, i.e. slightly lower levels than those effective in the dietary study. As for the males, the difference in sensitivity between the dietary study and the current findings may be due to different exposure routes or to higher sensitivity with continued exposure of pups in the dietary study.

Generally, it is expected that perinatal exposure to estrogenic compounds will lead to earlier development and increased growth of the prepubertal mammary glands [22;29;35], but no indications of progressed prepubertal mammary development were seen following mixed phytoestrogen exposure. Reduced scores for density in PND 13 females in the lowest mixture dose group may be a chance finding as no other changes in prepubertal mammary development or in expression of estrogen responsive genes were observed for males or females.

4.3 Mammary tumor latency

In the current study, mixed phytoestrogen exposure did not appear to alter latency to mammary tumors induced by the carcinogen MNU or to affect the number of TEBs on PND13. It has previously been proposed that SECO and other lignans may reduce the mammary cancer risk [8], while other studies have shown that exposure to flax seed containing SECO during early development enhanced mammary growth [9], increased the susceptibility to carcinogen-induced mammary tumors [10], or reduced TEB numbers suggesting a protective effect against mammary

tumors [36]. The timing of exposure appears important for the observed effects of phytoestrogens, but also differences in the specific compound studied (SECO, SECO diglucoside, flax seed or mixed phytoestrogen exposure) may account for the different results obtained. In agreement with the lack of change in tumor latency in the current study, Fritz et al., 1998, found an increased number of tumors, but no change in tumor latency following perinatal genistein exposure of rats [37]. Other studies showed that neonatal, but not prepubertal exposure to genistein increased tumor latency [2].

4.4 Male reproductive system

Genistein exposure, but not mixed phytoestrogen exposure, appeared to alter plasma testosterone levels in prepuberty in this study. The finding of reduced plasma testosterone levels in the genistein group at PND 13 but not at PND 22 may be a transient effect disappearing after the end of dosing or a chance finding. The reduction of testosterone by genistein confirms findings by Akingbemi et al., 2007, who found impaired testicular steroidogenesis in rats exposed perinatally to a soy-based diet containing genistein and daidzein [38]. They found that serum testosterone levels were decreased in pre-puberty and elevated in adulthood, possibly due to changes in Leydig cell numbers and function. In contrast, a rat study on perinatal exposure to soy beans showed increased Leydig cell numbers and increased testicular testosterone production in prepuberty [39]. No changes in adult serum testosterone levels were observed in the current study, and no changes in testicular histology were observed. As testosterone measurement is very variable, further examination such as quantitative examination of Leydig cells would be very relevant to include.

Unexpectedly, no changes in prostate weights were seen for genistein or for the phytoestrogen mixture. In general, it is known that estrogenic compounds may increase or decrease prostate

weights depending on dose level and age of examination [40-42], and possibly depending on the selected strain of rats. Genistein may also induce histological changes in prostates, but this was not examined in the current study [5].

Prostate development is known to be influenced by anti-androgens as well as estrogens [43]. Changes in mRNA levels of the AR regulated genes TRPM-2 and PBPC3 in ventral prostate have been observed following perinatal exposure to anti-androgenic chemicals in previous studies [44-47], but in the current study no changes in mRNA levels of the AR or androgen-regulated genes (TRPM-2, PBPC3) were found in prepubertal or adult ventral prostates.
Estrogenic compounds may alter expression of ER responsive genes such as IGF-1 [43], and reduced ERα mRNA and protein levels have been found in adult rat prostates following perinatal soy exposure [38], but no changes in IGF-1 or ERα mRNA expression of the prepubertal or adult ventral prostate were seen in this study. Another study in mice exposed to genistein in the neonatal period showed decreased ERα and AR levels (mRNA and protein) in adult testes [48], but no changes in these genes were observed in adult testes in the current study.

The absence of effects on testicular and prostatic gene expression in genistein exposed animals in the current study may be due to differences in dose levels and dosing periods, but examination of e.g. prostate histology, semen quality or epididymal changes would be required to exempt the phytoestrogen mixture for adverse effects on male reproductive organs.

4.5 Combination effects and human intake

Genistein, daidzein and SECO were present at relatively high levels in the mixture (9, 5, and 82% of the mixture, respectively), and it is likely that the combined exposure to these three compounds

have caused the observed effects on mammary glands and AGD. Overall, steroid synthesis inhibition and aromatase induction by lignans appear to be a plausible mechanism behind the AGD reduction observed in the current study, and may also be involved in the tendencies towards low estradiol levels in dams and female offspring.

Effects on AGD and male mammary hypertrophy were statistically significant at 100-fold "high human dietary intake" of phytoestrogens, while trends towards effects on these and other endpoints were seen also at 1x and 10x "high human dietary intake". Large variations made it difficult to determine whether the 1x or the 10x "high human dietary intake" dose can be considered a no-effect level in rats.

When considering the relevance for humans with an *average* phytoestrogen intake, it should be noted that the mean human dietary intake of phytoestrogens is far below the high dose level in this study. Furthermore, there may be differences between the study population of elderly men on which these "high human dietary intake" levels are based, and the pregnant women who are in focus when comparing with effects on reproductive development in perinatally exposed rats. Recent data on dietary phytoestrogen intake in German women (median age 63 years) [49] describe median intake levels 100-fold below median levels of the study population of Hedelin et al. [11], meaning that the lowest mixture dose (1x "high human dietary intake") is approximately equal to 100-fold mean human dietary intake level in the German female study population. At the 1-fold mixture dose level, the calculated intake of SECO is 104 mg/day per person corresponding to 38 g flaxseed per day [50]. Intake above this level is not expected from dietary sources, but intake of dietary supplements containing SECO could potentially lead to higher intake levels. Genistein intake at this "high human dietary intake" is 12 mg/day per person corresponding to 24 g soy beans per day; an intake level that may be exceeded by some consumers as NTP uses a high genistein intake of 25.8 mg/person per day [2].

The 10x “high human dietary intake” dose corresponding to 1000-fold “mean human exposure level” was a no-effect level in this study, and the safety margin can be considered sufficient for pregnant women with average phytoestrogen intakes. It should be noted that only a limited amount of data on phytoestrogen intake can be found in the literature, and that intake data for pregnant women are scarce. An excessive human intake of phytoestrogens may result from e.g. dietary supplements or special dietary habits. Such intakes may contribute to endocrine disruption particularly for sensitive subpopulations or for persons exposed in combination with other endocrine disrupting chemicals.

Further studies on male and female mammary glands and on male nipple retention and AGD may be required to determine no-effect levels for particularly SECO. This study showed no effects on prostate or testicular weights and no changes in mRNA levels of steroid receptors and their target genes, but further studies on e.g. prostate histology, semen quality or epididymal changes would be beneficial. Experimental and epidemiological studies on the influences of the dietary sources of SECO (flax seed, rye bread) on reproductive development would also be very relevant.

4.6 Conclusions

Perinatal exposure to high doses of phytoestrogens can induce adverse mammary changes in rats, particularly in males. This is seen by increased hypertrophic growth and increased alveolar secretion as a sign of feminization. These changes are likely related to the endocrine disruption reflected by changes in male AGD.

Flaxseed and rye bread are the main dietary sources of lignans including SECO [11], and although they are often associated with beneficial health effects it may be necessary to examine further whether realistic intakes of these dietary components also have adverse effects on male

reproductive development. This study indicated adverse effects at high intake levels in rats, but does not provide evidence for risk of phytoestrogen-mediated endocrine disruption at normal human dietary consumption levels. Further studies are warranted to increase the knowledge upon which risk assessment on dietary phytoestrogen exposure during pregnancy and infancy is based. However, an excessive human intake of phytoestrogens may result from e.g. dietary supplements or special dietary habits and may contribute to endocrine disruption, particularly for sensitive subpopulations or for persons exposed in combination with other endocrine disrupting chemicals.

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Figure legends:

Figure 1. Anogenital distance in male offspring of rats perinatally exposed to a mixture of phytoestrogens or genistein. Data points show litter means, horizontal lines are means of litter means ±SD. * Indicates statistically significant difference from controls ($p<0.05$, including all data points).

Figure 2. Hormone levels in dams and offspring exposed to a mixture of phytoestrogens or genistein, mean±SEM. * indicates a statistically significant difference compared to controls in an ANOVA followed by Dunnett's test.

Figure 3. Histopathology of mammary glands of male rats perinatally exposed to a mixture of phytoestrogens. A, control mammary gland with absence of secretion in alveoli. B, hypertrophy in male mammary gland from the highest dose-group (Mix-213). C, apocrine-like changes and secretion filled alveoli (arrow) in male mammary gland from the highest dose-group (Mix-213). Prevalence of males with secretory material in alveoli (D), hypertrophy (E) or apocrine-like changes (F). *: $p<0.05$ compared to controls. Scale bar in lower left corner of pictures represents 50µm. 40x magnification.

Figure 4. Histopathology of mammary glands of female rats perinatally exposed to a mixture of phytoestrogens. A, tubuloalveolar pattern in control female mammary gland. B, lobuloalveolar pattern in mammary gland of female from the highest dose-group (Mix-213). C, prevalence of females with lobuloalveolar pattern. Scale bar in the bottom right corner of photos represents 50µm. 40x magnification.

Figure 5. Cumulated incidence of mammary tumors in rats exposed to a mixture of phytoestrogens or genistein (n=14-16). No statistically significant differences between controls and exposed groups were observed.

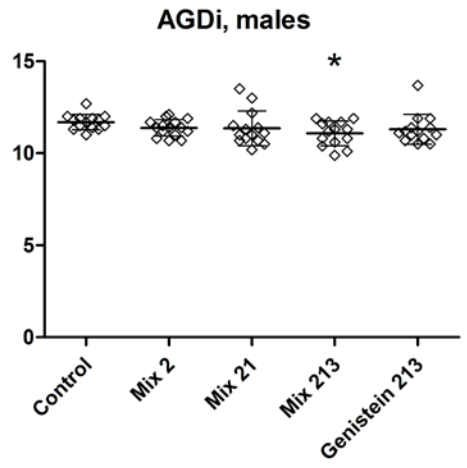

Figure 1.

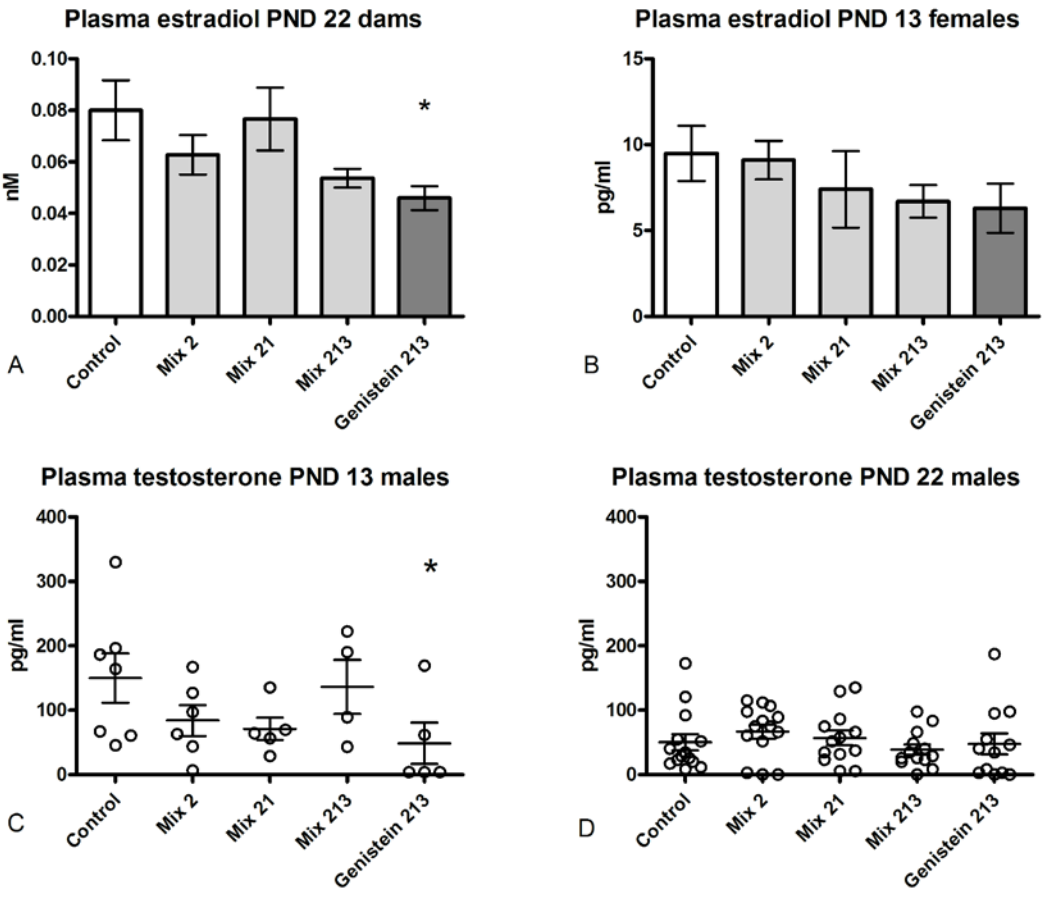

Figure 2.

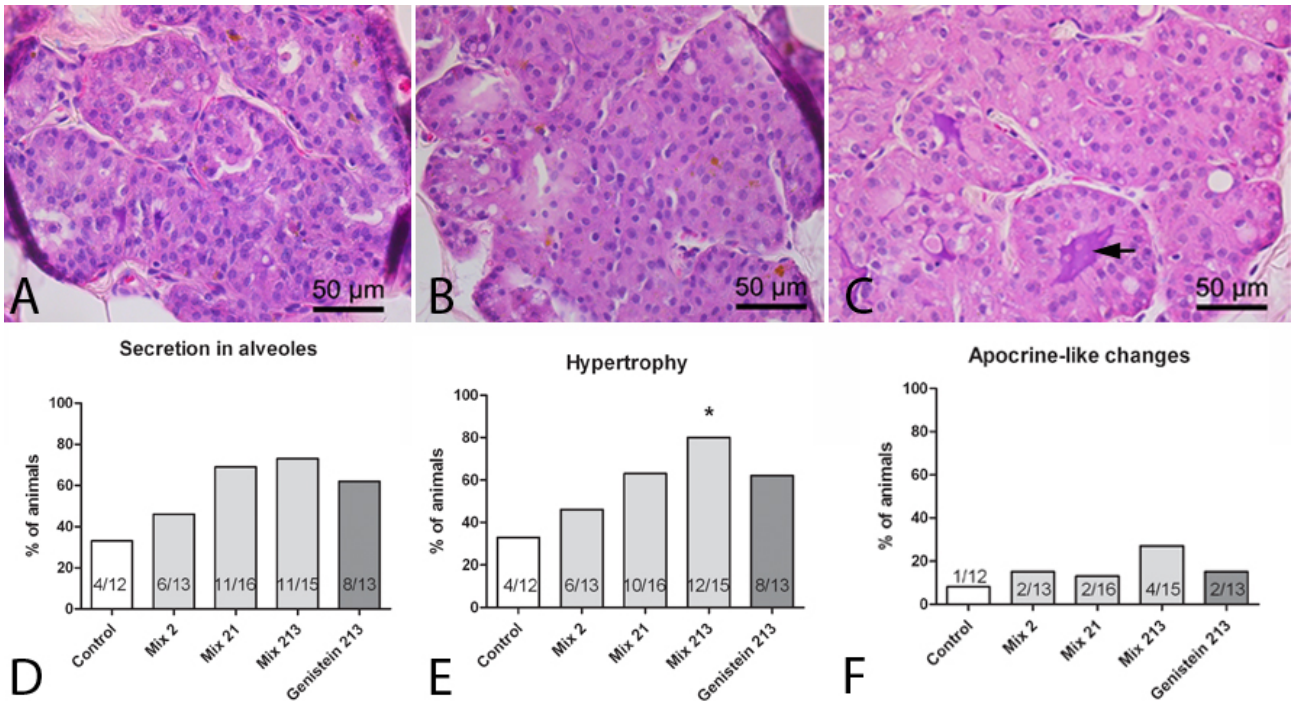

Figure 3.

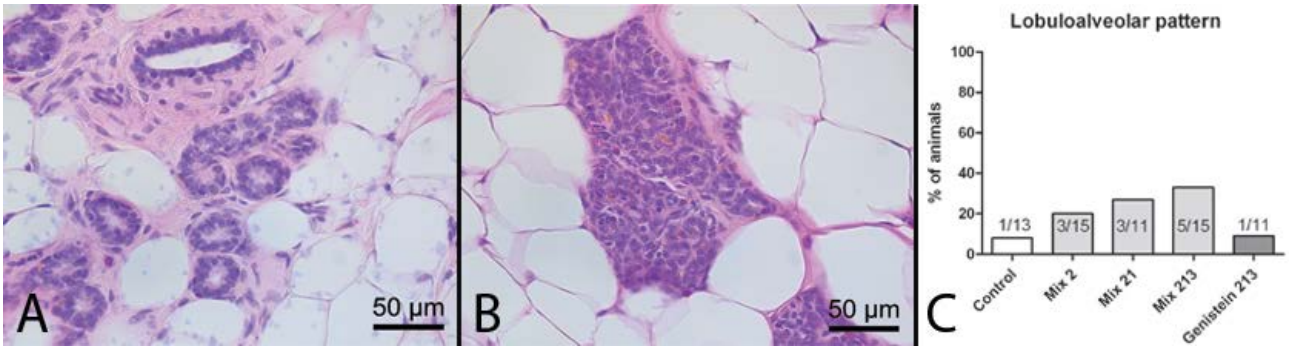

Figure 4.

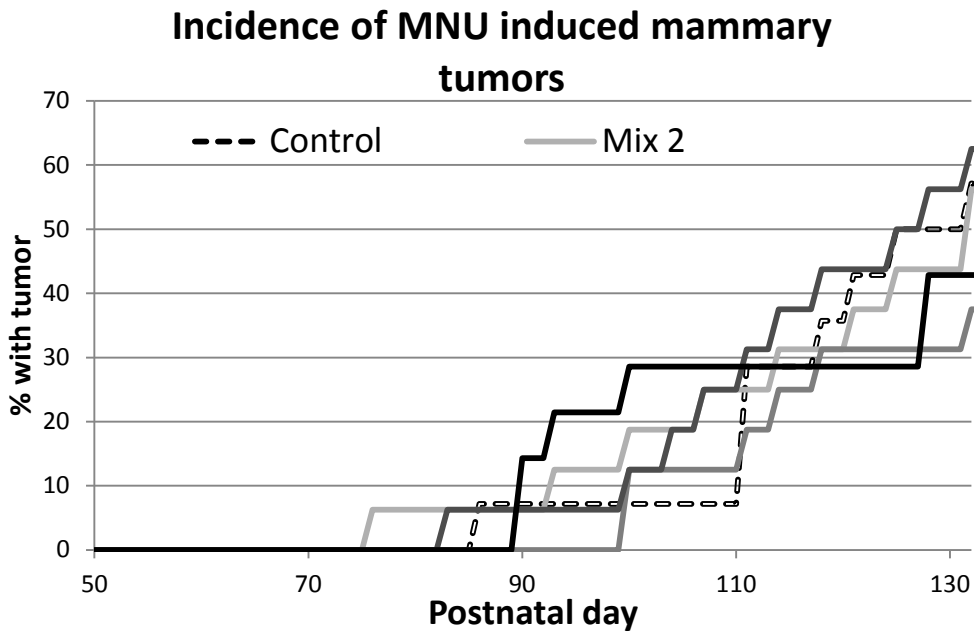
Incidence of MNU induced mammary tumors
Control
Mix 2
% with tumor
0
10
20
30
40
50
60
70
50
70
90
110
130
Postnatal day

Figure 5.

Tables

Table 1. Composition of phytoestrogen mixture based on “high human dietary intake” from Hedelin et al.,2006 [11]. The gray-shaded row indicates the total intake in mg/kg bw/day for each dose groups and corresponds to the group names “Mix 2”, “Mix 21” and “Mix 213”

	1x high human dietary intake, µg/kg/day	**10x high human dietary intake, µg/kg/day**	**100x high human dietary intake, µg/kg/day**
#Genistein	194	1940	19400
#Daidzein	123	1230	12300
#Equol	0.22	2.2	22
#Formononetin	0.28	2.8	28
#Biochanin A	0.28	2.8	28
*Enterolactone	2.78	27.8	278
*Enterodiol	0.01	0.1	1
Coumestrol	0.08	0.8	8
*Secoisolarici resinol (SECO)	1739	17390	173900
*Matairesinol	6.82	68.2	682
*Lariciresinol	40.2	402	4020
*Pinoresinol	19.07	190.7	1907
Total (µg/kg/day)	**2125.7**	**21257.4**	**212574**
Total (mg/kg/day)	**2.13**	**21.3**	**212.6**
Total isoflavonoids and isoflavonoid metabolites (#) (mg/kg/day)	**0.3**	**3.2**	**31.8**
Total lignans and lignan metabolites (*) (mg/kg/day)	**1.8**	**18.1**	**180.8**

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

	Control	**Mix 2**	**Mix 21**	**Mix 213**	**Genistein 213**
No. of dams (litters)	16 (14)	16 (15)	16 (14)	16 (14)	16 (14)
Maternal weight gain GD7-GD21	92.0 ± 8.7	89.4 ± 17.8	98.8 ± 22.6	88.6 ± 11.0	83.1 ± 13.7
Maternal weight gain GD7-PD1	14.4 ± 12.3	8.7 ± 18.1	14.6 ± 22.7	2.6 ± 18.0	2.5 ± 16.1
Maternal weight gain PD1-22	6.4 ± 13.9	8.1 ± 14.4	13.2 ± 15.2	13.9 ± 18.5	18.8 ± 13.7
Gestation length (days)	23.0 ± 0.1	22.9 ± 0.3	23.0 ± 0.0	22.9 ± 0.2	23.0 ± 0.3
% post-implantation loss	6.1 ± 8.5	2.3 ± 4.2	6.0 ± 7.5	5.2 ± 5.7	7.6 ± 14.8
% perinatal loss	8.7 ± 10.0	6.6 ± 9.9	7.7 ± 8.5	10.4 ± 10.0	11.4 ± 18.8
Live pups per litter	11.6 ± 1.9	13.1 ± 1.4	12.3 ± 1.7	12.6 ± 2.3	11.4 ± 3.2
% Postnatal death	2.7 ± 6.5	4.5 ± 8.4	1.8 ± 3.6	5.4 ± 9.9	5.4 ± 9.9
% Males	46.3 ± 11.2	47.5 ± 16.4	52.0 ± 18.5	48.0 ± 13.7	47.0 ± 14.5
Birth weight male offspring	6.8 ± 0.3	6.6 ± 0.3	6.7 ± 0.4	6.5 ± 0.7	6.7 ± 0.5
Birth weight female offspring	6.5 ± 0.2	6.0 ± 0.5	6.2 ± 0.4	6.0 ± 0.5	6.3 ± 0.4

Table 3.
Reproductive organ weights (absolute and relative) and serum hormone levels in rat offspring exposed to a mixture of phytoestrogens or to genistein from GD 7 to PND 13. Mean ±SD

		Control	Mix 2	Mix 21	Mix 213	Genistein 213
PND 13, males	**N (N prostate)**	14 (9)	15 (13)	14 (12)	14 (13)	14 (12)
	Body weight, g	26.7±3.0	24.5±3.5	24.8±3.0	24.5±4.3	25.1±3.6
	Testes, mg	68.7±6.1	66.5±5.8	65.0±6.1	64.1±9.4	67.1±8.5
	Ventral prostate, mg	6.0±2.2	5.2±1.4	5.5±1.7	4.4±1.5	5.5±1.4
	Liver, mg	693±100	642.1±81.6	625±85	638±142	654±125
	Rel. testes, mg/g	2.60±0.26	2.75±0.36	2.64±0.19	2.63±0.20	2.68±0.17
	Rel. ventral prostate, mg/g	0.15±0.12	0.18±0.09	0.19±0.10	0.17±0.08	0.19±0.09
	Rel. liver, mg/g	26.03±2.46	26.41±2.90	25.30±2.25	26.00±2.51	25.93±2.52
	Plasma testosterone[#], pg/ml	150±102	84±58	71±39	136±84	**47±73***
	Testis testosterone, ng/testis	0.78±0.45	0.47±0.59	0.70±0.71	0.65±0.43	0.49±0.37
PND 13, females	**N**	14	13	15	14	14
	Body weight, g	25.8±3.2	22.9±3.3	23.7±2.7	24.4±4.2	24.4±3.4
	Liver, mg	673±89	612±103	628±92	641±149	622±126
	Rel. liver, mg/g	26.1±2.2	26.8±2.2	26.4±1.8	26.1±2.3	27.0±2.2
	Plasma estradiol[#]	9.5±3.6	9.1±2.5	7.4±5.0	6.7±2.1	6.3±3.2
PND 22, males	**N**	14	14	13	13	12
	Plasma testosterone, pg/ml	50.4±47.3	66.9±40.2	56.8±41.6	38. 5±28.6	47.5±55.7
PND 22, females	**N**	14	14-15	10	13	13
	Body weight, g	50.1±6.0	46.4±5.1	46.7±4.1	46.8±7.1	48.6±7.7
	Uterus, mg	24.1±5.3	23.0±2.8	23.4±2.9	24.2±5.5	22.9±2.7
	Ovaries, mg	17.8±2.5	17.0±2.7	16.7±2.1	16.5±2.9	17.8±3.4
	Rel. uterus, mg/g	0.48±0.10	0.50±0.07	0.50±0.06	0.52±0.12	0.48±0.09
	Rel. ovaries, mg/g	0.36±0.04	0.37±0.05	0.36±0.04	0.35±0.04	0.37±0.05
	Plasma estradiol pg/ml	15.9±4.1	17.8±3.3	18.3±3.3	17.6±4.2	17.1±3.8
PND 150, males	**N**	14	15-16	15-16	16	13-14
	Body weight, g	460±28	454±37	464±36	436±35	448±36
	Testes, mg	3878±354	3775±260	3784±193	3708±372	3683±283
	Ventral prostate, mg	617±151	592±113	639±160	605±135	616±148
	Rel. testes, mg/g	8.4±0.7	8.3±0.7	8.0±1.2	8.5±0.6	8.3±0.6
	Rel. ventral prostate, mg/g	1.3±0.3	1.3±0.3	1.4±0.4	1.4±0.3	1.3±0.5
	Plasma testosterone pg/ml	2576±2360	1777±2673	1372±1102	1694±1720	1949±1692
PND 135 females	**N (N proestrus/estrus) [##]**	10 (9)	16 (12)	14 (10)	16 (14)	14 (9)
	Body weight, g	244±16	249±14	251±22	250±22	252±22
	Uterus, mg	881±363	843±436	811±277	797±413	876±187
	Ovaries, mg	88±16	85±13	87±11	95±13	100±16
	Rel. uterus, mg/g	3.6±1.5	3.4±1.7	3.4±1.2	3.2±1.7	3.6±0.8
	Rel. ovaries, mg/g	0.36±0.08	0.34±0.05	0.36±0.04	0.38±0.05	0.41±0.06
	Plasma estradiol pg/ml	15.2±7.9	26.6±16.1	21.0±12.6	26.5±13.5	21.8±9.5

[#] at PND 13 samples for plasma hormone levels were pooled for 2-3 pups, n=4-7 for males and n=5 for females. [##]In adult females, plasma estradiol levels, uterus and ovary weights were compared for animals in proestrous or estrous only. * indicates a statistically significant difference from controls in an ANOVA followed by Dunnett's test.

Supplementary data description

Supplementary data contain tables with information on probe/primer sequences for mRNA expression analyses (Table S1) and data on mammary whole mount evaluation and tumor histology (Table S2).

Table S1. Information on probe/primer sequences

Gene name	Probe/primer sequences
β-actin	Probe (reporter-nucleotide sequence-quencher): 5′-FAM-TAT GGA ATC CTG TGG CAT CCA TGA AAC TAC A-TAMRA-3′ Forward:, 5′-ATG CCC CGA GGC TCT CTT-3′ Reverse: 5′-CAA CGT CAC ACT TCA TGA TGG A-3′
18s rRNA	Probe: 5′-FAM-ACC GGC GCA AGA CGA ACC AGA G-TAMRA-3′ Forward:, 5′-GCC GCT AGA GGT GAA ATT CTT G-3′ Reverse: 5′-GAA AAC ATT CTT GGC AAA TGC TT-3′
TRPM-2	Probe: 5′-FAM-AGT TTC TGA ACC AGA GCT CAC CCT TCT ACT TCT G-TAMRA-3′ Forward: 5′-CTG GTT GGT CGC CAG CTA GA-3′ Reverse: 5′-ATG CGG TCC CCG TTC AT-3′
IGF-1	Probe: 5′-FAM-CAA CAC TCA TCC ACA ATG CCC GTC T-TAMRA-3′ Forward: 5′-GAC CAA GGG GCT TTT ACT TC-3′ Reverse: 5′-GCA GCG GAC ACA GTA CAT CT-3′
AR	Probe: 5′-FAM-TCG CGA TTC TGG TAT GCT GCT GC-TAMRA3′ Forward: 5′-GAC ACT TGA GAT CCC GTC CT-3′ Reverse: 5′-GAG CGA GCG GAA AGT TGT AG-3′
PBP C3	Probe: 5′-FAM-TCATCT AGA ATA CTG CAG CCA GAA CCA CTG G-TAMRA-3′ Forward: 5′-CCA TCC CCA TTT GCT GCT AT-3′ Reverse: 5′-AGT CAC AGT TGA GTT AAT TGT ACC TCT AAT AAC-3′
ERα	Probe: 5′-FAM-CCA CCC TGC TGG TTC A-MGB-3′ Forward: 5′-ATT CCT TCC TTC CGT CTT ACT GTC T-3′ Reverse: 5′-AGC CGC CGA GGT ACA GAT T-3′
ERβ	Probe: 5′-FAM-CCA CTA AGC TTC CTC TTC AGT GTC TCT CTG TTT ACA-TAMRA-3′ Forward: 5′- TTG GTG TGA AGC AAG ATC ACT AGA G-3′ Reverse: 5′- AAC AGG GCT GGC ACA ACT G-3′

Table S2. Mammary gland changes in rats perinatally exposed to a mixture of phytoestrogens or to genistein including mammary tumor histology in rats exposed to MNU at PND 50. TEBs: terminal end buds. Values are mean ±SD for density scores, areas (mm^2), and TEB number. For tumors, numbers are given as: affected animals/total number of animals (% affected animals in parentheses). *: statistically significant compared to controls with $p<0.05$.

		Control	Mix 2	Mix 21	Mix 213	Genistein 213
Mammary whole mounts						
Females PND 13	Density	2.8±0.6	**2.0±0.6***	2.1±0.5	2.4±0.9	2.5±0.9
	Area	50±12	41±10	40±10	45±11	46±14
	TEBs	5.7±4.9	9.3±7.6	6.8±9.1	7.7±13.3	9.2±7.1
Males PND 13	Density	2.8±0.8	2.4±0.7	2.7±0.6	2.6±0.9	2.4±0.7
	Area	42±10	40±7	41±10	38±12	40±11
	TEBs	4.4±4.8	8.1±14.1	7.0±8.6	10.8±12.7	7.0±9.0
Females PND22	Density	2.5±0.9	2.3±0.8	2.5±0.8	2.8±0.6	3.0±0.6
	Area	93±22	86±26	68±22	95±30	91±29
Females PND135	Density	2.9±0.9	3.3±1.3	2.9±1.4	2.9±1.3	3.3±1.0
Males PND150	Density	2.3±1.0	2.1±0.8	2.8±0.9	2.9±1.4	2.6±1.4
Mammary tumors in MNU exposed females						
Dominating histopathological type	Cribriform	9/9 (100%)	6/11 (55%)	4/7 (57%)	9/11 (82%)	6/7 (86%)
	Papillary	0/9 (0%)	5/11 (45%)	2/7 (29%)	2/11 (18%)	1/7 (14%)
	Comedo	0/9 (0%)	0/11 (0%)	1/7 (14%)	0/11 (0%)	0/7 (0%)

PAPER 4

Pernille Rosenskjold Jacobsen, Marta Axelstad, Julie Boberg, Louise Krag Isling, Sofie Christiansen, Karen Riiber Mandrup, Line Olrik Berthelsen, Anne Marie Vinggaard, Ulla Hass.

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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.

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

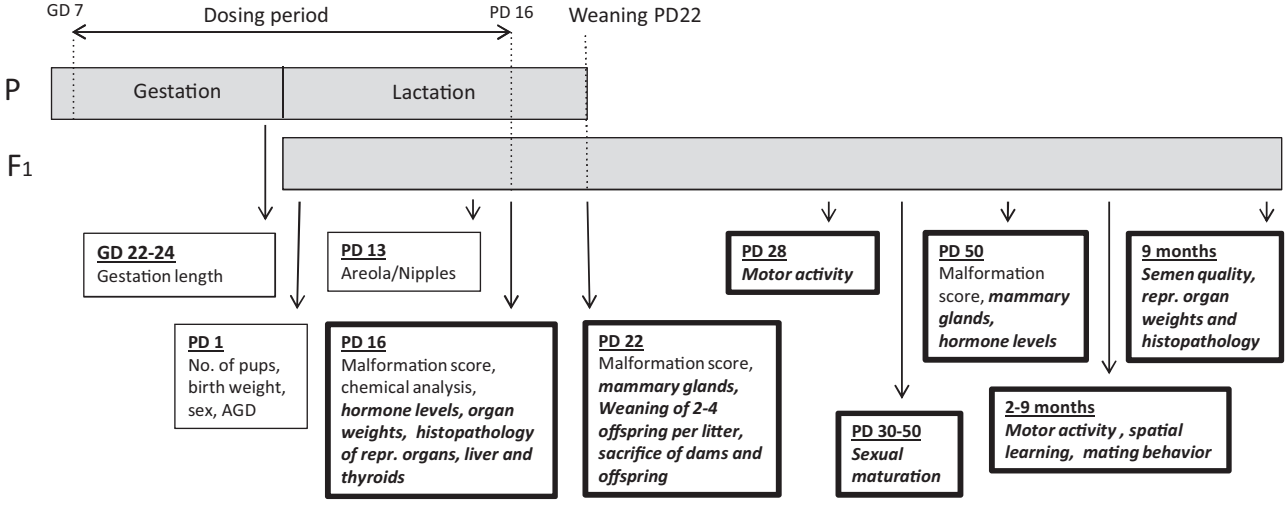

Fig. 1. Schematic overview of the design of the mixture study. Pregnant dams were dosed from gestation day 7 to postnatal day 16 with a mixture of pesticides, or the individual chemicals. In the present paper the results from organ weights, histopathology, mammary glands, hormone levels, timing of sexual 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.

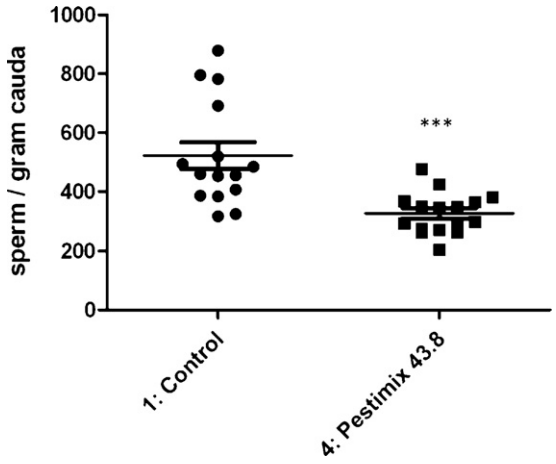

Fig. 2. Number of sperm per gram cauda epididymis in the adult males from the control group, and the group dosed with the highest dose of the pesticide mix during gestation and lactation. The control group included measurements from 15 males (representing 13 litters) and the mixture group included data 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.

* $p < 0.05$.

** $p < 0.01$.

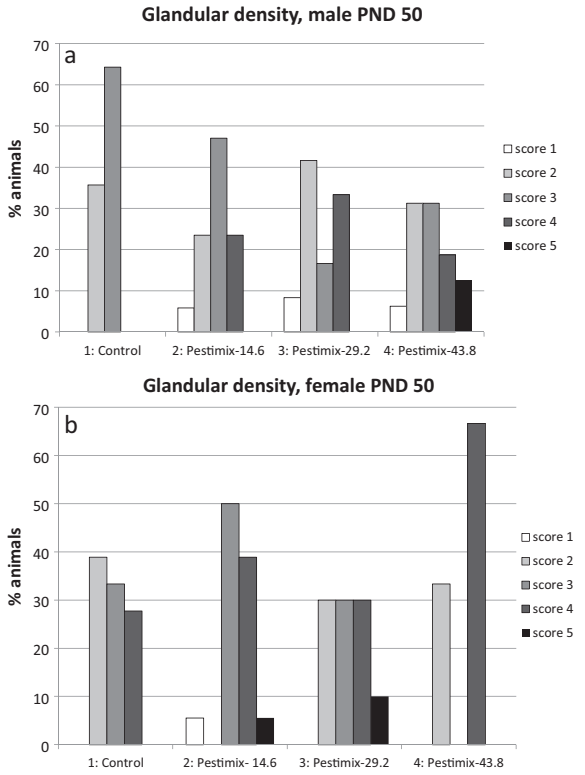

Fig. 3. (a,b) Mammary gland whole mounts PD 50. Glandular density was evaluated as described in Section 2 and the distribution of density scores is presented as % of animals with a given score for each group separately. For males, scores 4 and 5 are only present in exposed groups indicating a dose-related 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.

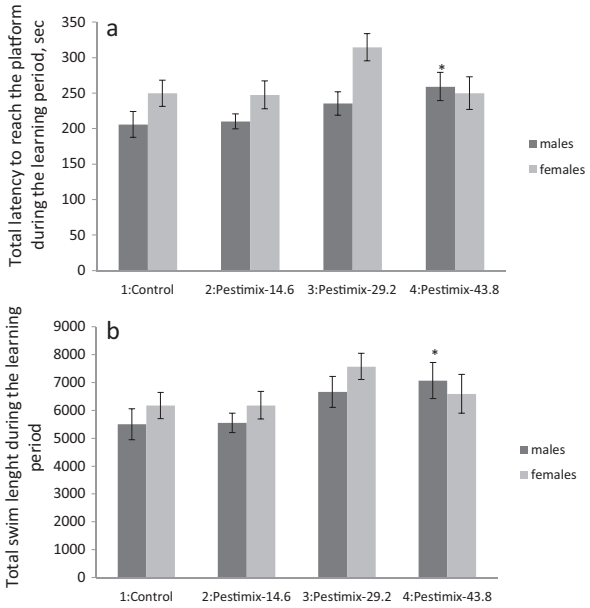

Fig. 4. (a,b) Total latency to reach the platform (a) and total swim length (b) in adult male and female offspring exposed to 0, 14.6, 29.2 or 43.8 mg/kg/day of the pesticide mixture during fetal and neonatal life. Data is shown as group means ± SEM for total latency and swim length to reach the platform 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

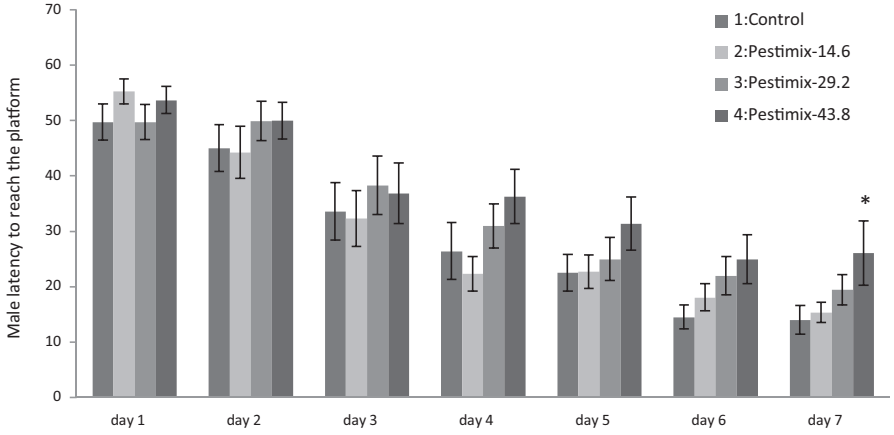

Fig. 5. Mean daily latency to reach the platform in adult male rat offspring exposed to 0, 14.6, 29.2 or 43.8 mg/kg/day of the pesticide mixture during fetal and neonatal life. Data is shown as group means ± SEM for latency in the Morris Water Maze for the first seven days of the learning testing, $n = 9–10$. 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.

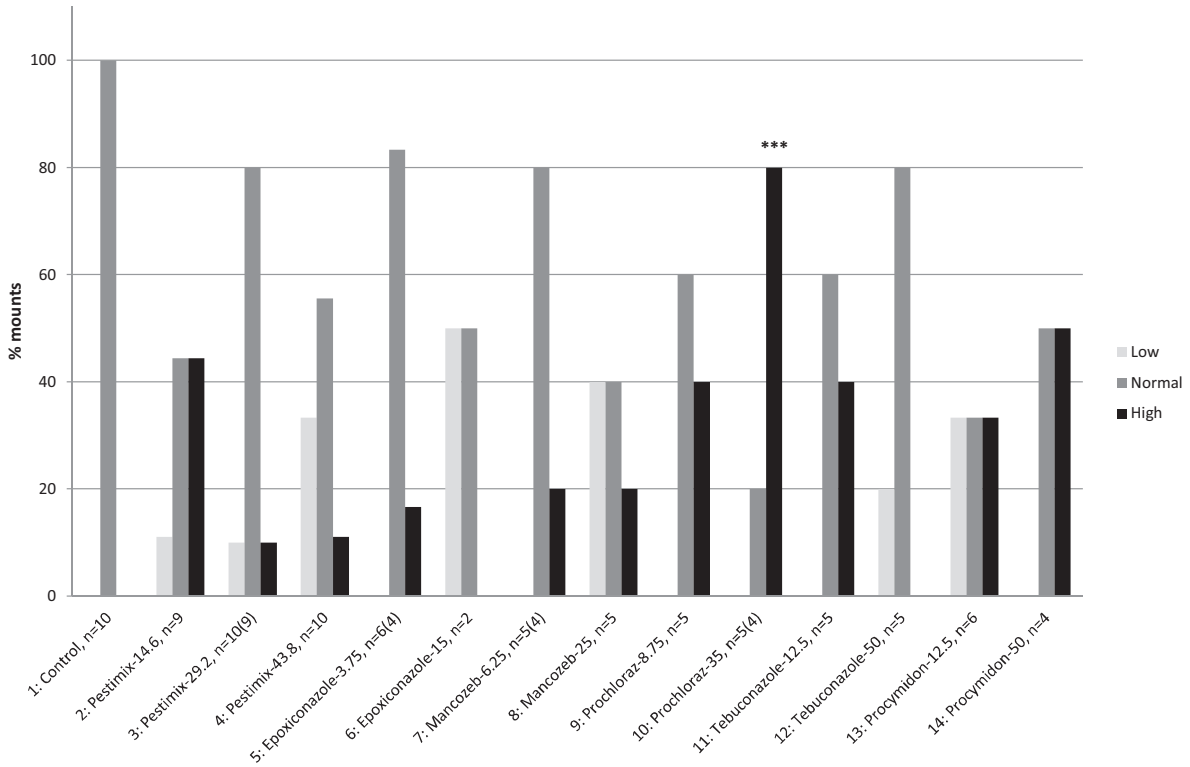

Fig. 6. Mating behavior, % mounting activity and distribution in 3 groups. Low (number of mounts ≤5), medium (number of mounts >5 and <30) or high (number of mounts ≥30), during a 20 min. period, shown as group means (%). The number of males is shown as *n*, while the number in parenthesis is the number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

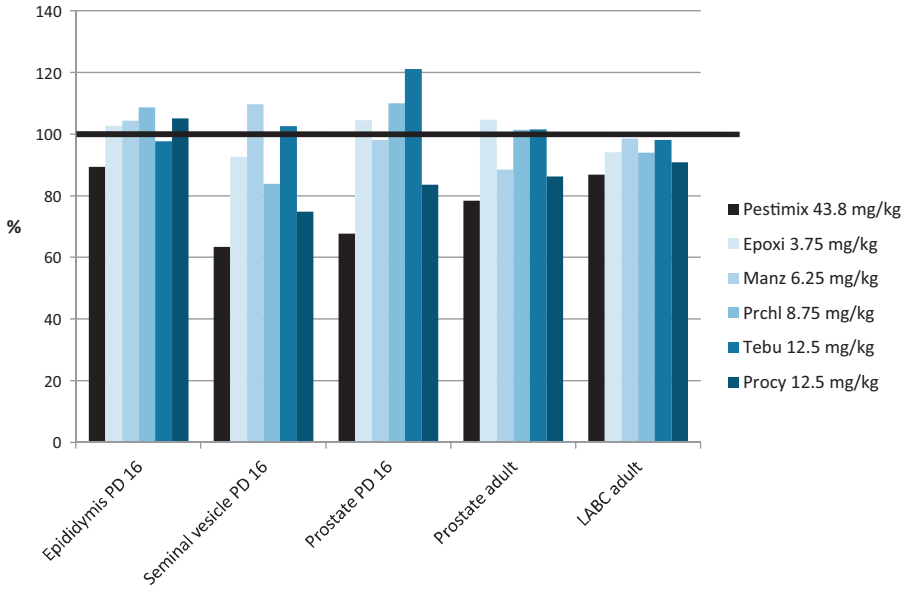

Fig. 7. Organ weight changes compared with controls in male rat pups exposed to the low dose of epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu), procymidone (Procy) or the highest mixture dose of these pesticides (Pestimix) from GD 7 to PND 16. Values are expressed as 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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Supplementary data

Table S1. Relative male organ weights on PD 16 and in adult rats (PD 260-280), exposed to the pesticides singly or in mixture during fetal and neonatal life.

Relative organ weights (mg/100g bw)	n	Testes	Epididymis	Prostate	Ves sem	LABC	Bulbo	Liver	Thyroid
Male offspring PD 16									
1: Control	15	337±23	71.2±23.2	34.5±6.8	33.8±8.5	89.1±20.6	5.7±1.2	2557±114	14.5±2.8
2: Pestimix-14.6	16	350±38	70.7±9.3	30.4±5.4	27.9±6.3	79.2±10.7	5.1±1.4	2546±105	14.0±2.0
3: Pestimix-29.2	9	362±14	60.8±25.4	28.2±5.6	28.5±4.7	86.6±16.1	4.9±1.1	2677±68	14.8±2.6
4: Pestimix-43.8	12	357±13	**63.7±6.5**. ##**	**23.4±5.9***. ###**	**21.5±7.9**. ##**	82.4±16.0	5.0±2.0	2645±140	14.3±4.8
5: Epoxi-3.75	6	348±34	73.2±8.8	36.1±7.3	31.3±6.5	98.7±19.5	7.3±2.9	2548±136	15.8±5.2
6: Epoxi-15	3	358±38	82.8±11.3	44.8±11.7	28.6±5.3	74.6±7.4	6.6±2.1	2682±152	12.9±3.3
7: Manz-6.25	5	351±17	74.4±8.4	33.9±4.1	37.1±5.3	82.2±5.1	5.9±0.8	2525±259	15.6±2.3
8: Manz-25	7	359±29	71.9±32.1	36.0±11.3	31.7±7.9	94.5±16.0	6.2±1.1	2540±93	15.8±3.7
9: Prchl-8.75	9	357±23	77.4±10.2	38.0±3.5	28.4±5.9	85.4±12.1	5.3±1.3	2531±152	11.3±4.7
10: Prchl-35	4	355±36	67.1±5.2	36.1±6.6	26.7±9.6	76.1±5.0	5.0±1.8	2520±148	11.3±1.9
11: Tebu-12.5	8	363±29	69.6±28.5	41.8±6.0	34.7±11.5	89.6±12.5	6.1±1.5	2599±70	14.2±3.3
12: Tebu-50	5	346±19	72.4±8.4	32.3±9.6	37.7±6.6	80.6±6.4	5.4±0.8	**2749±121# #**	17.2±3.1
13: Procy-12.5	6	**376±22*, ##**	74.8±11.2	28.8±5.1	25.3±4.5	93.3±21.0	5.1±0.5	2636±128	14.3±3.0
14: Procy-50	3	363±27	67.2±12.9	23.3±1.8	29.5±2.6	41.7±36.2	2.4±2.2#	2722±260	15.2±2.5
Adult male offspring									
1: Control	16	792±63	142±15	129±31	391±71	283±36	44±14	2673±190	4.5±0.6
2: Pestimix-14.6	18	854±78	152±16	147±39	428±76	279±43	53±17	2547±148	5.3±2.2
3: Pestimix-29.2	12	861±143	158±21	134±25	455±99	275±43	38±13	2551±131	5.3±1.0
4: Pestimix-43.8	16	865±63	150±17	101±43	444±75	246±44	46±12	2590±174	4.1±1.2
5: Epoxi-3.75	10	**860±65#**	141±16	135±26	421±88	267±43	46±10	2549±208	4.2±0.6
6: Epoxi-15	2	(970±65)	(180±13)	(155±42)	(502±37)	(307±4)	(71±19)	(2747±223)	(5.6±0.7)
7: Manz-6.25	8	833±90	153±19	114±50	390±89	280±56	48±11	2604±89	6.2±3.7
8: Manz-25	10	817±80	151±12	126±25	405±100	259±19	43±14	2694±195	6.0±3.2
9: Prchl-8.75	10	846±70	146±18	131±32	419±62	266±35	56±13	2610±216	4.4±0.6
10: Prchl-35	6	844±39	147±10	148±29	447±53	287±26	52±25	**2431±180#**	4.6±0.4
11: Tebu-12.5	8	860±91	154±27	131±31	407±92	278±35	39±14	2523±144	4.6±0.3
12: Tebu-50	8	803±65	138±15	116±28	407±113	259±48	33±2	2632±176	5.1±0.9
13: Procy-12.5	8	844±113	154±28	111±32	410±77	258±60	38±15	2567±148	4.3±0.7
14: Procy-50	6	840±48	150±12	**90±38#**	363±48	241±28	42±13	**2374±255#**	4.2±0.7

Epoxiconazole (Epoxi), mancozeb (Manz), prochloraz (Prchl), tebuconazole (Tebu) or procymidone (Procy).Ves.sem.: seminal vesicle; Bulbo: glandula bulbocavernosus; LABC: levator ani/bulbospongiosus 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 ANOVA of relative weights 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.
Data for adults in the Epoxi-15 group are in parentheses as these are left out of statistical analysis due to only 2 animals in this group.

Table S2. Mammary gland whole mounts and histology PND 22 and 50, in rats exposed to the pesticide mixture during fetal and neonatal life.

		1: Control	2: Pestimix-14.6	3: Pestimix-29.2	4: Pestimix-43.8
Mammary whole mounts PD 22, males	n	14	15	8	10
	Density score	2.9 ± 1.1	2.1 ± 0.8	2.6 ± 1.3	2.1 ±1.4
	Longitudinal growth, mm	10.0 ± 1.3	9.8 ± 2.3	9.5 ± 1.9	10.4 ± 1.9
	Transverse growth, mm	5.5 ±1.1	5.5 ±1.4	5.7 ±1.3	5.1 ± 0.9
	Area, mm^2	41.9 ± 11.9	43.5 ± 16.7	41.7 ± 13.3	43.2 ± 15.7
	TEB number	6 ± 4.5	7.6 ± 11.6	5.1 ± 2.0	6.3 ± 6.5
Mammary whole mounts PD 22, females	N	10-11	13-15	4-5	10
	Density score	3.5 ± 1.0	3.0 ± 0.9	3.2 ± 0.4	3.5 ± 0.8
	Longitudinal growth, mm	11.2 ± 1.7	10.3 ± 1.5	11.0 ±1.9	11.2 ± 1.9
	Transverse growth, mm	5.7 ± 1.3	5.6 ± 1.3	6.4 ± 2.2	5.9 ± 1.0
	Area, mm^2	49.4 ± 14.4	46.3 ± 13.4	54.5 ± 26.2	49.3 ± 12.2
	TEB number	24.6 ±19.5	21.0 ± 13.9	36.0 ± 15.9	20.5 ± 16.9
Mammary whole mounts PD 50, male	n litters [total number of examined animals]	13 [13-14]	15 [15-16]	8 [9-10]	13 [13-14]
	Density score	2.7 ± 0.5	2.8 ± 0.9	2.9 ± 1.0	2.9 ±1.1
	Longitudinal growth, mm	15.4 ± 2.9	16.3 ± 2.3	**18.7* ± 4.1**	16.6 ± 2.5
	Transverse growth, mm	9.9 ± 1.3	9.5 ± 2.2	10.4 ± 1.8	9.9 ± 2.9
	Area, mm^2	114.7 ± 33.5	117.5 ± 35.0	138.7 ± 44.7	126.2 ± 40.4
Mammary whole mounts PD 50, females	n litters [total number of examined animals]	15 [18]	16 [18]	8 [10]	7 [9]
	Density score	2.8 ± 0.8	3.4 ± 0.9	3.3 ± 1.0	3.4 ±1.0
Mammary histology PD50, males	Secretory material in ducts	17% (1/6)	-	-	33% (3/9)
	Vacuolization of mammary epithelium	50% (3/6)	-	-	67% (6/9)
Mammary histology PD 50, females	Presence of lobuloalveolar pattern, % (number affected/total)	0% (0/14)	8% (1/13)	11% (1/9)	8% (1/12)

Data on whole mounts is shown as group means ± standard deviation. At PD 22, mammary gland from one pup per litter was examined, n is the number of examined litters per dose group. At PD 50, one or two pups per litter were examined and values are based on litter means, and in brackets following the n for number of litters, the total number of examined animals is given. Histology data are given as % of affected animals and in parentheses are given (numbers affected of animals/total number of animals examined). Statistically significant differences between controls and exposed groups are marked in bold. Asterisks indicate significance levels: * $p<0.05$.

Table S3. Total motor activity levels in adult male and female rat offspring exposed to the pesticides singly or in mixture during fetal and neonatal life.

	Male		Female	
	no. animals (litters)	activity count + std. dev.	no. animals (litters)	activity count + std. dev.
1:Control	16(14)	977+138	18(15)	1339+79
2:Pestimix-14.6	18(17)	1285+97	18(17)	1591+110
3:Pestimix-29.2	12(9)	1137+175	10(8)	1145+82
4:Pestimix-43.8	16(14)	1142+128	12(11)	1463+65
5:Epoxiconazol-3.75	10(6)	1210+184	10(7)	1355+164
6:Epoxyconazol-15	2(2)	1227+374	6(4)	1460+154
7:Mancozeb-6.25	8(5)	1153+65	8(5)	1518+264
8:Mancozeb-25	10(7)	1068+137	10(7)	**1896+193****
9:Prochloraz-8.75	10(9)	1293+156	10(9)	**1718+181***
10:Prochloraz-35	6(4)	1522+129	6(4)	1634+184
11:Tebuconazol-12.5	10(8)	1121+113	8(7)	**1938+135****
12:Tebuconazol-50	8(6)	980+104	8(6)	1524+221
13:Procymidon-12.5	8(7)	1261+92	8(7)	1545+178
14:Procymidon-50	6(4)	772+81	6(4)	1295+85

Data is shown as group means + standard deviation, and for each dose group the tested number of animals and the number of litters they represent is shown. Statistically significant differences between controls and exposed groups are marked in bold. Asterisks indicate significance levels: * $p<0.05$, ** $p<0.01$. An overall significant differences between males and females was seen in the data ($p<0.0001$). Statistically significant differences between males and females from the same dose group were seen in groups 1, 2, 4, 7, 8, 9, 11, 12 and 14 but are not marked in the table.

Table S4. Swim lengths and latencies in male and female rat offspring exposed to the pesticides singly or in mixture during fetal and neonatal life.

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MALE		Swim length								Swim latency							
	n	day 1	day 2	day 3	day 4	day 5	day 6	day 7	total	day 1	day 2	day 3	day 4	day 5	day 6	day 7	total
1: Control	10 (10)	1416+ 319	1411+ 458	880+ 459	650+ 468	562+ 329	312+ 168	273+ 117	5504+ 1760	50+ 10.4	45+ 13.4	34+ 16.3	26+ 16.3	23+ 10.5	15+ 7.0	14+ 8.3	206+ 57
2: Pestimix-14.6	10 (10)	1642+ 184	1358+ 445	867+4 85	514+2 63	518+2 22	356+ 175	299+ 129	5553+ 1096	55+ 7.2	44+ 14.8	32+ 15.9	22+ 9.9	23+ 9.6	18+ 7.8	15+ 5.8	210+ 34
3: Pestimix-29.2	10 (9)	1481+ 261	1608+ 367	1154+ 496	829+4 02	631+3 65	514+ 321	448+ 263	6665+ 1662	50+ 9.5	50+1 0.6	38+ 15.8	31+ 12.0	25+ 11.6	22+ 10.3	19+ 8.2	235+ 50
4: Pestimix-43.8	10 (10)	1571+ 312	1544+ 384	1056+ 571	911+4 34	785+4 07	580+ 436	627 +604	**7074+ 2052***	54+ 7.9	50+ 10.5	37+ 17.4	36+ 15.4	31+ 15.2	25+ 14.0	**26+ 18.3***	**259+ 63***
5: Epoxi-3.75	6(4)	1561+ 152	1289+ 375	1127+ 466	625+2 76	718+3 73	532+ 248	366+ 145	6218+ 1393	50+ 3.6	40+ 10.8	38+ 14.4	27+ 9.3	29+ 11.7	25+ 10.6	18+ 6.3	227+ 46
6: Epoxi-15	2(2)	1450+ 158	1378+ 335	824+9 04	492+3 53	456+2 94	301+ 87	259+ 109	5159+ 2241	54+ 9.2	46+ 2.2	30+ 25.5	20+ 9.9	20+ 8.8	15+ 4.1	12+ 2.8	196+ 63
7: Manc-6.25	5(4)	1351+ 374	1509+ 381	873+3 77	668+4 15	428+1 31	464+ 217	296+ 162	5589+ 809	44+ 13.5	45+ 11.6	29+ 12.8	26+ 13.8	16+ 4.0	20+ 10.2	14+ 7.7	194+ 33
8: Manc-25	5(5)	1642+ 273	1254+ 314	1007+ 256	610+2 35	557+1 86	415+ 213	339+ 127	5824+ 944	58+ 5.4	41+ 9.1	38+ 8.2	26+ 7.6	23+ 7.4	21+ 6.2	16+ 5.0	222+ 27
9: Prchl-8.75	5(5)	1558+ 207	1353+ 375	932+4 96	450+1 84	514+2 71	340+ 106	347+ 154	5494+ 1164	57+ 4.7	47+ 12.5	36+ 16.3	23+ 10.1	23+ 12.6	20+ 5.0	23+ 10.7	231+ 51
10: Prchl-35	5(4)	1626+ 239	1363+ 298	983+3 04	559+1 21	486+2 18	321+ 180	298+ 181	5637+ 657	54+ 7.4	43+ 9.4	34+ 10.4	23+ 5.6	23+ 10.1	15+ 8.1	12+ 5.9	204+ 30
11: Tebu-12.5	5(5)	1778+ 328	1708+ 356	1500+ 477	1074+ 620	1024+ 836	916+ 698	**756+ 493***	**8756+ 2924****	56+ 5.3	51+ 8.8	50+ 11.3	37+ 16.8	37+ 22.6	38+ 21.4	**31+ 14****	**300+ 78****
12: Tebu-50	5(5)	1515+ 129	1536+ 615	882+6 29	642+4 35	718+6 83	376+ 311	293+ 106	5962+ 1868	50+ 3.2	50+ 16.7	33+ 18.4	27+ 16.0	28+ 23.7	18+ 11.8	14+ 5.1	220+ 54
13: Procy-12.5	6(6)	1572+ 396	1478+ 520	739+2 14	471+3 03	563+2 22	319+ 164	290+ 145	5432+ 949	52+ 5.7	48+ 10.5	29+ 8.2	20+ 8.8	24+ 8.2	18+ 10.4	15+ 6.6	204+ 28
14: Procy-50	4(4)	1480+ 88	1449+ 280	1190+ 171	886+1 73	663+4 91	573+ 222	598+ 260	6839+ 1074	55+ 6.7	50+ 6.5	46+ 10.1	35+ 7.0	30+ 20.5	30+ 8.5	**31+ 10****	**277+ 26****

FEMALE		Swim length								Swim latency							
	n	day 1	day 2	day 3	day 4	day 5	day 6	day 7	total	day 1	day 2	day 3	day 4	day 5	day 6	day 7	total
1: Control	10 (10)	1593+ 192	1222+ 357	911+ 346	586+ 235	669+ 398	617+ 305	574+ 277	6175+ 473	56+ 5.7	45+ 11.9	35+ 12.9	26+ 9.5	30+ 15.7	28+ 12.8	26+ 13.9	249+ 58
2: Pestimix-14.6	10 (10)	1469+ 373	1215+ 271	891+3 39	640+2 82	553+3 81	674+ 416	741+ 355	6184+ 497	52+ 11.3	45+ 11.6	35+ 11.5	28+ 10.0	25+ 14.6	28+ 15.7	30+ 11.8	247+ 62
3: Pestimix-29.2	10 (8)	1540+ 233	1413+ 382	1156+ 331	918+ 417	877+ 408	894+ 419	775+ 425	7578+ 496	53+ 6.7	51+ 11.2	50+ 11.5	41+ 16.9	43+ 18.0	40+ 16.1	34+ 15.9	314+ 58
4: Pestimix-43.8	10 (10)	1484+ 257	1246+ 397	1017+ 582	890+ 352	753+ 436	698+ 502	505+ 299	6597+ 738	51+ 7.3	44+ 12.1	36+ 16.5	35+ 12.9	29+ 15.6	29+ 17.6	23+ 14.0	250+ 73
5: Epoxi-3.75	6(4)	1559+ 144	1241+ 419	1041+ 516	953+ 484	918+ 638	883+ 578	868+ 562	7466+ 3159	53+ 6.2	46+ 15.3	41+ 16.0	38+ 16.5	34+ 18.5	34+ 20.7	33+ 18.4	283+ 105
6: Epoxi-15	6(4)	1602+ 307	1225+ 398	793+ 291	896+ 283	522+ 85	702+ 206	600+ 98	6342+ 158	55+ 4.4	44+ 15.4	33+ 13.7	37+ 14.3	21+ 4.7	29+ 6.9	25+ 6.3	248+ 11
7: Manc-6.25	5(4)	1507+ 104	1491+ 275	1187+ 322	1400+ 84	933+ 255	800+ 253	822+ 201	8143+ 611	52+ 6.2	49+ 10.6	43+ 16.0	52+ 5.6	35+ 12.9	33+ 9.5	34+ 11.2	300+ 33
8: Manc-25	5(5)	1518+ 175	1476+ 402	999+ 779	1049+ 808	677+ 358	778+ 484	500+ 284	7001+ 1767	53+ 4.7	47+ 9.5	31+ 8.5	33+ 21.6	28+ 15.1	28+ 14.8	19+ 9.4	243+ 46
9: Prchl-8.75	5(5)	1654+ 433	1395+ 613	569+ 349	405+ 199	433+ 214	766+ 466	575+ 450	5801+ 1852	51+ 9.5	45+ 18.9	24+ 20.5	17+ 9.9	18+ 9.1	29+ 16.3	24+ 18.0	211+ 77
10: Prchl-35	5(4)	1450+ 249	1224+ 265	789+ 462	633+ 356	639+ 344	470+ 352	382+ 178	5590+ 1150	55+ 6.8	47+ 13.7	33+ 11.8	27+ 13.7	31+ 15.7	21+ 16.1	17+ 9.0	234+ 51
11: Tebu-12.5	5(4)	1786+ 194	1511+ 141	1314+ 86	905+ 242	810+ 235	821+ 232	1043+2 81	8191+ 790	59+ 0.9	54+ 5.9	50+ 8.8	36+ 9.6	33+ 7.9	35+ 8.7	41+ 9.1	310+ 21
12: Tebu-50	5(5)	1429+ 273	1101+ 469	1065+ 312	876+ 466	650+ 560	916+ 581	846+ 546	6885+ 2832	52+ 11.0	40+ 15.2	44+ 13.4	33+ 12.6	29+ 19.5	36+ 17.0	33+ 17.5	270+ 87
13: Procy-12.5	6(6)	1598+ 253	1418+ 377	1246+ 436	930+ 634	584+ 124	653+ 320	474+ 303	6906+ 1761	56+ 4.9	47+ 11.4	44+ 15.0	32+ 16.6	25+ 6.1	29+ 13.1	22+ 13.7	256+ 61
14: Procy-50	4(4)	1217+ 435	1501+ 482	1005+ 219	565+ 145	666+ 276	595+ 281	626+ 93	6179+ 438	44+1 7.8	49+1 2.4	37+ 6.9	36+ 17.8	36+ 20.6	33+ 20.5	32+ 17.7	270+ 82

Data is shown as group means + standard deviation. Statistically significant differences between controls and exposed animals for post hoc analysis with all 14 dose groups are marked in bold. Asterisks indicate significance levels: * $p<0.05$, ** $p<0.01$. An overall statistically significant differences between males and females was seen in the data on days 4-7 ($p<0.0001$).

PAPER 5

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Mammary gland effects of perinatal exposure to environmentally relevant endocrine disrupting chemicals.

Draft manuscript/ in preparation

Mammary gland effects in mixture studies with endocrine disrupting chemicals

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Keywords: endocrine disruption, mammary gland, mix, paracetamol, whole mount

Abstract

Endocrine disrupting chemicals (EDCs) such as estrogenic and anti-androgenic compounds may affect mammary gland development. Estrogenic chemicals have been shown to increase mammary gland development in females, however, little is known on the effects of anti-androgens and mixtures of EDCs with dissimilar modes of action.

Pregnant Wistar rat dams were exposed to paracetamol or mixtures of environmentally relevant EDCs with estrogenic (Emix), anti-androgenic (Amix) or dissimilar modes of action (TotalMix) of 100-, 200- or 450-fold high end human intake estimates during gestation and the lactational period. Mammary glands of prepubertal and adult female and male offspring were examined for endocrine disrupting effects.

Emix and TotalMix were found to increase mammary outgrowth prepubertally in females. No effects of anti-androgens were observed. In adulthood, morphological changes of female mammary glands may indicate a higher prevalence of prolactin producing pituitary tumours in the TotalMix group compared to controls.

The present study showed estrogenic effects on mammary glands, but no NOAEL was determined for changes in prepubertal female mammary glands. Further studies on dose-levels are needed for risk assessment of the effects of perinatal estrogenic chemicals on mammary glands.

Introduction

Sexual differentiation during fetal development and infancy is highly dependent on hormonal influence. Disturbance of the hormonal balance by endocrine disrupting chemicals may have important impact on the development of a wide range of hormone sensitive organs in the young animal or child. In rodent studies, perinatal exposure to such chemicals has been shown to affect offspring and some changes are persistent in adulthood [Jacobsen et al. 2012; Soto et al. 2008]. During sexual differentiation *in utero* the mammary glands are formed. Androgens are responsible for the male-like differentiation, blocking the nipple formation [Kratochwil 1977; Sourla et al. 1998] and estrogens are important for the female development of mammary glands [Borellini and Oka 1989; Sourla et al. 1998]. Several environmental chemicals have been demonstrated to have estrogenic or anti-androgenic properties and may thus interfere with mammary gland development.

Estrogenic chemicals have been shown to affect mammary gland development in females and to accelerate mammary gland growth. In rat studies, an increase in the area and the number of terminal end buds (TEBs) have been shown in prepubertal female mammary glands when exposed to estrogenic compounds [Cotroneo et al. 2002; Delclos et al. 2001; Murrill et al. 1996]. Moreover, studies have shown increased lobular or alveolar hyperplasia [Biegel et al. 1998; Delclos et al. 2001; Masutomi et al. 2004; Takagi et al. 2004] and secretory dilation of alveoli of adult female mammary glands exposed to estrogens [Biegel et al. 1998; Foster et al. 2004]. Terminal end buds (TEBs) are undifferentiated proliferative structures in rat mammary glands where neoplastic transformation primarily occurs, and an increase in the number of TEBs may be a sign of increased risk of mammary cancer later in life [Russo et al. 1990]. Lobules are differentiated structures and lobule development with alveolar or lobular hyperplasia, on the other hand, may be a sign of accelerated growth or increased differentiation of the mammary gland. Estrogens have also been shown to affect male mammary glands, increasing the area, density and branching of the gland [Mandrup et al. 2012; Wang et al. 2006; You et al. 2002], suggesting an enhanced growth of mammary glands in males.

Anti-androgenic chemicals are well-known to affect male offspring. For example, phthalates are known to induce malformations of the sexual organs, change the ano-genital distance and affect nipple retention [Christiansen et al. 2012; Parks et al. 2000; Schultz et al. 1999]. However, the possible effects of anti-androgens on mammary gland development have only been described in a few studies. Saad et al. (2011) observed increased branching in whole mounts of pubertal female

mammary glands following perinatal exposure to vinclozolin [Saad et al. 2011]. In a study by Jacobsen et al (2012), no change in density was observed in either male or female offspring PD22 exposed perinatally to anti-androgenic pesticides. No changes in outgrowth has been reported in whole mounts of prepubertal mammary glands exposed to anti-androgens [Jacobsen et al. 2012; Saad et al. 2011]. Histology of adult males exposed perinatally to di-*n*-butyl phthalate was reported by Lee et al (2004) to show increased incidence of vacuolar degeneration in alveolar cells and alveolar atrophy in the mammary glands [Lee et al. 2004]. Moreover, developmental exposure to a mixture of anti-androgenic pesticides in a study by Jacobsen et al (2012) showed effects indicating an increased feminization of adult male mammary glands [Jacobsen et al. 2012]. Thus, anti-androgens appears to affect both female and male mammary glands, however further studies are needed for more insights on the influence of anti-androgens on mammary gland development.

Humans are exposed to a wide range of chemicals with estrogenic and anti-androgenic properties. To mimic human exposure, an environmentally relevant mixture of EDCs with dissimilar modes of action was studied for effects on male and female mammary development of perinatally exposed rats. This paper describes the effects of a mixture of chemicals known to be mainly estrogenic (Emix), a mixture of chemicals known to be mainly anti-androgenic (Amix) and a total mixture of both the estrogenic and the anti-androgenic compounds. Additionally, paracetamol has been shown to have endocrine disrupting capacities and was tested alone and included in the total mixture (TotalMix). The TotalMix included the following 13 chemicals: the phthalates di-*n*-butyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP), the pesticides vinclozolin, prochloraz, procymidone, linuron and epoxiconazole, the pesticide metabolite p,p'-DDE, the UV-filter substances octyl methoxycinnamate (OMC) and 4-methyl-benzylidene camphor (4-MBC), the phenolic compound bisphenol A, the preservative butyl paraben and the analgesic compound paracetamol. This mixture affected a range of anti-androgenic endpoints during early development as reported previously (Christiansen et al., 2012).

As noted, mammary effects of endocrine disrupting chemicals have been described in both prepubertal and adult rats [Delclos et al. 2001; Murrill et al. 1996; Wang et al. 2006; You et al. 2002]. In rodent studies, examination of the entire mammary gland during development is possible by evaluation of mammary whole mounts, i.e. mammary tissue fixed on a glass slide and stained for microscopic investigation.To determine the optimal age for evaluation of the mammary gland whole mounts, we performed an initial study (study A) comparing results of neonatal, prepubertal

and adult mammary whole mount evaluation following exposure to the TotalMix. Female and male mammary gland whole mounts of offspring were evaluated for outgrowth, density and number of TEBs. In the following study (Study B), whole mounts of prepubertal animals were prepared and the effect of each sub-component of the TotalMix (Emix, Amix, paracetamol) was compared to the effect of the TotalMix. Histological sections of adult mammary glands were evaluated for adult offspring. Other reproductive parameters will be described in another paper.

Materials and methods

Chemicals

In these two studies, dose levels were selected based on human intake data. A detailed description of the TotalMix composition and choice of dose-levels can be found in Christiansen et al. [Christiansen et al. 2012]. The highest dose of the TotalMix (TotalMix-450) represented 450-fold "high human intake levels" of each compound. Paracetamol was present at a dose level of 360 mg/kg bw/day in the TotalMix-450, a dose level of paracetamol that corresponds to approximately 7-fold human intake levels in individuals consuming 6 paracetamol tablets of 500mg per day. Study B also included the same dose of paracetamol in the dose-group receiving paracetamol only (PM-360). Study B also included 100- and 200- fold "high human intake levels" for the total mixture (TotalMix-100 and TotalMix-200), and 200-fold and 450-fold "high human intake levels" for the anti-androgen (Amix-200 and Amix-450) as well as the estrogen (Emix-200 and Emix-450) sub-mixtures. The ratios and amounts of the single compounds in the mixtures are shown in table 1.

Table 1. Chemicals included in the mixture, mixture ratio equivalent to estimated human exposure levels and dose levels of the mixture used in rats for the studies A and B. Doses are in mg/kg bw/day.

Group and dosing	1. Control	2. Mix-100	3. Mix-200	4. Mix-450	5. Amix-200	6. Amix-450	7. Emix-200	8. Emix-450	9. Paracetamol
DBP	0	1	2	4.5	2	4.5	0	0	0
DEHP	0	2	4	9	4	9	0	0	0
Vinclozolin	0	0.9	1.8	4.05	1.8	4.05	0	0	0
Prochloraz	0	1.4	2.8	6.3	2.8	6.3	0	0	0
Epoxiconazole	0	1	2	4.5	2	4.5	0	0	0
Procymidone	0	1.5	3	6.75	3	6.75	0	0	0
Linuron	0	0.06	0.12	0.27	0.12	0.27	0	0	0
ppDDE	0	0.1	0.2	0.45	0.2	0.45	0	0	0
4-MBC	0	6	12	27	0	0	12	27	0
OMC	0	12	24	54	0	0	24	54	0
Bisphenol A	0	0.15	0.30	0.675	0	0	0.30	0.675	0
Butyl paraben	0	6	12	27	0	0	12	27	0
Paracetamol	0	80 *	160 *	360 *	0	0	0	0	360*

*: For group 2-4, mixtures contain paracetamol on GD 13-19 and PND 14-22. Group 9 received paracetamol on GD 13-19 and PND 14-22 and vehicle on other days.

Animals and experimental design

Two studies were performed using comparable study designs. Time-mated nulliparous female Wistar rats were supplied on gestation day (GD) 3 and on GD4 dams were assigned to dose groups with similar weight distributions in all groups. Dams were dosed by gavage from GD7 to GD21. The day after expected delivery was defined as pup day (PD) 1. Dosing of dams was resumed during lactation PD1-22. However, when animals were exposed to paracetamol, dosing of paracetamol was restricted to GD13-GD19 and PD14-22. Animals were administered acidified tap water (in glass bottles) and soy- and alfalfa free diet (Altromin 1314, GmBH, Lage Germany) *ad libitum*. Animals were housed in semi-transparent polycarbonate cages (15x27x43cm) with Aspen bedding (Tapvei, Brogaarden, Gentofte, Denmark) in controlled environmental conditions with 22°C ± 1°C, 10 air changes per hour, air humidity 55% ± 5 and 12h light-dark cycles with lights on from 9PM to 9AM. Until GD 17, pregnant rat dams were housed in pairs and thereafter alone. 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 of the National Food Institute at the Technical University of Denmark.

The first series of studies (Study A) aimed to determine the optimal timing of mammary examination in whole mounts, and mammary effects were examined in neonatal (PD 6), prepubertal (PD 22) and post-pubertal (PD 49 or 55) offspring exposed to the mixture. In the second study (Study B), the same mixture was subdivided into anti-androgens, estrogens and paracetamol, and mammary effects at PD22 of each sub-mixture were compared with effects of the total-mixture.

Study A was performed in two blocks. In each block 28 dams were assigned to 2 groups (n=14): control (0mg/kg) and TotalMix-450. In study B 152 dams were assigned to 9 groups (n=20 in control and TotalMix-100 groups and n=16 in each of the other groups): control (0mg), 3 TotalMix groups (TotalMix-100, TotalMix-200 and Totalix-450), 2 Amix groups (Amix-200 and Amix-450), 2 Emix groups (Emix-200 and Emix-450) and PM-360. The dose-levels used in the two studies are described in table 1.

Necropsy

Pups were anesthetized with CO_2/O_2 and sacrificed by decapitation. In study A, pups were sacrificed at different time points: neonatally, prepubertally and post-pubertally. In the first block, one male and one female pup were sacrificed PD6 and one male and one female were weaned on PD22 for sacrifice PD49 (females) or PD55 (males). In the second block, one female and one male were sacrificed on PD22. In study B, one male and one female pup were sacrificed on PD22. At sacrifice, the 4^{th} mammary gland was dissected from male and female pups for whole mounting. Furthermore, 1 male and 1 female in study B were weaned on PD22. At 10 months (males) and 13 months (females), the weaned offspring were sacrificed in CO_2/O_2 anesthesia and the 4^{th} mammary gland and uterus were dissected and fixed in formalin for histology. Adult females were sacrificed when in estrous verified by macroscopic evaluation of unstained smears (clots of vaginal material on glass slide) and confirmed by microscopic evaluation of uterus.

Whole mounts

Mammary glands were dissected and spread on a glass slide, covered with parafilm and pressed while drying for approximately 2 hours. The mammary glands were fixed in 4% formalin buffer, stained with alum carmine, dehydrated in alcohol and cleared with xylene before mounting with a

coverslip. Whole mounts were scanned in a flatbed scanner (4800dpi). Measurements were performed on the digital images in Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). Spatial calibration was performed for each picture before measurements were made, and the data were collected in a Microsoft Excel spread sheet. Mammary glands PD22 were evaluated for outgrowth (outer area, transverse growth, longitudinal growth and distance to the lymph node, and in study B additionally the distance to the 5th gland), number of TEBs and density (score 1-5 describing the level of branching and budding, where score 5 has extensively branching and budding). At PD6 and in males PD55, same parameters were evaluated, except the distance to the lymph node and the distance to the 5th gland. Female mammary glands PD49 were often incomplete due to the large size of the gland and only the density was scored in a triangular area defined by the nipple and the lymph nodes. Density score criteria were adjusted for age and gender and thus not directly comparable between age and gender. In study B, mammary gland whole mounts were evaluated for controls and the high-dose groups (TotalMix-450, Amix-450, Emix-450 and PM-360). As effects were seen in the high-dose Emix group, the lower dose (Emix-200) was also evaluated.

Histology

Mammary glands from study B were sectioned and stained in haematoxylin and eosin. Histologic evaluation was performed on controls and the high-dose groups (TotalMix-450, Amix-450, Emix-450 and PM-360). Female mammary glands were evaluated for lobuloalveolar pattern, dilation of and secretion in alveoli and ducts and hyperplasia of alveolar and duct epithelium. Histological slides of male mammary glands were evaluated for secretory activity (dilated lumens with secretory material and vacuolated alveolar epithelium) and hypertrophy of mammary epithelium. Histological parameters evaluated were scored on a two- or three-scaled score. The stage of estrous cycle of adult females at necropsy was confirmed by histological evaluation of uterus. Females not confirmed to be in estrous or proestrous were not examined for these changes.

Statistics

Statistical analysis was performed in the statistical software SAS Enterprise Guide 3.0 and 4.3 (SAS Institute Inc., Cary, NC, USA) or GraphPad Prism 5. The alpha level was set at 0.05 and the litter

was the statistical unit. Continuous data from males and females were pooled and gender was analysed for interaction with dose-groups. If no interaction was present, data were analysed with an analysis of variance (ANOVA) with body weight (bw) as a covariate and gender as a main factor. However, if an interaction was present, data for each gender were analysed separately. Data were evaluated for normal distribution and homogeneity of variance before performing the statistical tests. Data not fulfilling criteria for homogeneity of variance or normal distribution (i.e. generally the density data, the distance to the lymph node and the number of TEBs) were analysed using a non-parametric statistical test (Kruskal-Wallis) with a Dunn's post-hoc test correcting for multiple comparisons when more than two groups were compared, or with a non-parametric t-test (Mann Whitney) if only two groups were compared. Histological data were analysed with a Fisher's exact test in GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, NC, USA).

Results

Whole mounts

In the first study, the effects of perinatal dosing on mammary gland development were investigated at three different ages. No statistically significant differences between control animals and rats exposed to TotalMix-450 were found (data not shown).

In the second study, a statistically significant decrease in the distance to the lymph node was observed in females PD22 exposed perinatally to Emix-450 compared to controls, indicating an increased outgrowth of the mammary gland (Figure 1A). A statistically significant decrease in the distance to the lymph node was additionally found for females in the TotalMix-450 group when data on the distance to the lymph node from females PD22 were pooled from the two studies. This may indicate a stronger effect of Emix-450 than of TotalMix-450 on mammary outgrowth, but there was no statistically significant difference between these two groups.

Due to the finding of increased outgrowth in the high-dose Emix group, re-evaluation of these whole mounts was performed including the low-dose Emix group (Emix-200). This evaluation revealed a statistically significant decrease in the distance to the lymph node in both Emix groups (Figure 1B, C, D). No difference in TEB numbers for females or males was found (data not shown).

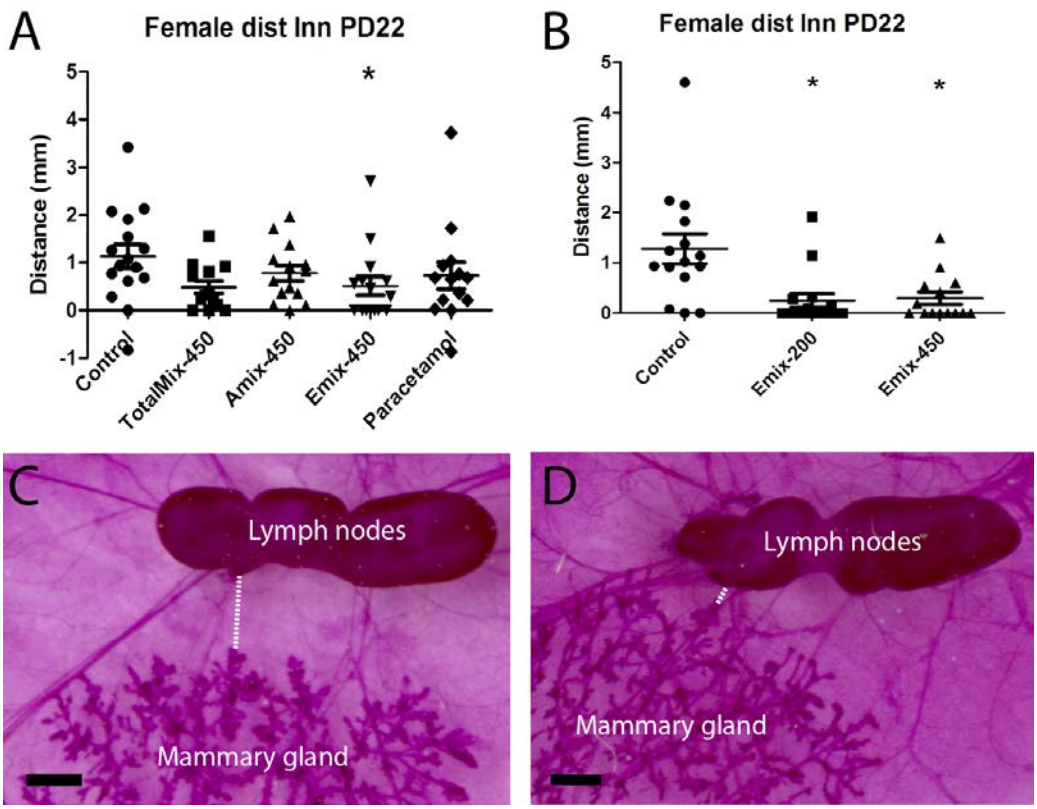

Figure 1. Distance to lymph node in study B. A, Distance to the lymph node in females PD22 from the high dose-groups compared to controls. B, Distance to the lymph node in females PD22 in the Emix groups compared to controls. C, Distance to the lymph node in a female whole mount from the control group PD22. D, Distance to the lymph node in a female whole mount from the Emix-200 group PD22. Dashed white lines illustrate the shortest distance from the mammary gland to the lymph nodes. Black bar in C and D represents 1mm. *: statistically significantly different from controls in Dunn's post-hoc test with $p<0.05$.

Histology

At 13 months of age, females with striking histological changes in parts of the mammary glands were observed. An increased prevalence of females showing simple cuboidal to low cuboidal epithelium, abundant amounts of secretory material in alveoli and extensive lobular development was observed in the TotalMix-450 group compared to controls ($p<0.05$) (Table 2). These focal changes resembled lactating glands and suggest an increased prevalence of prolactin producing pituitary tumours in exposed females compared to controls. Females not showing signs of effects from a prolactin producing pituitary tumour, showed no significant changes in lobuloalveolar

pattern, secretory activity or hyperplasia of mammary epithelium (data not shown). Male mammary glands showed no statistically significant changes in the prevalence of secretory activity or hypertrophy of the mammary epithelium at 10 months of age (data not shown). More than half of the animals showed hypertrophic mammary epithelium in all groups and the highest incidence (81%) of males with hypertrophic epithelium was present in the Emix-450 group. Some of the male mammary glands with hypertrophic epithelium were extensively hypertrophic with abundant eosinophilic cytoplasm, similar to apocrine-like changes described by Boberg et al (submitted 2013). Apocrine-like changes were also observed in controls and the biological relevance of such changes are yet unknown.

Table 2. Histological changes in mammary glands of adult females (13 months old) and males (10 months old).

	Control	**TotalMix-450**	**Amix-450**	**Emix-450**	**Paracetamol**
Females (n)	17	16	16	16	13
Well-developed lobules with dilated and secretion filled alveoli with simple cubic or low cubic epithelium	1	6*	3	3	3
Males (n)	16	15	16	16	11
Hypertrophic epithelium (including apocrine-like changes)	10	8	9	13	6
Apocrine-like changes	3	3	0	5	0

*: statistically significant in a Fisher's exact test with $p<0.05$.

Discussion

Study A: Age for evaluation of mammary gland whole mounts

Study A showed no statistically significant effects. Effects PD22 were observed in our previous studies on ethinyl estradiol [Mandrup et al. 2012] and other studies have shown effects of EDCs in mammary glands PD22 [Fritz et al. 1998; Murrill et al. 1996]. Additionally, most parameters were not possible to evaluate in whole mounts of adult female mammary glands and may prevent the detection of relevant changes in the mammary glands. Outgrowth parameters and the number of TEBs were not possible to evaluate and the density was measured in a defined area known to

belong to the 4th gland. Hence, it was decided to examine mammary glands on PD22 in study B for evaluation of different combinations of mixtures of endocrine disrupting chemicals.

Study B: Estrogenic compounds influence mammary development

Perinatal exposure to a mixture of endocrine disrupting chemicals gave rise to morphological changes in mammary gland whole mounts prepubertally, but not in histological sections of mammary glands in adult rats. Females PD22 exposed perinatally to the mixture of estrogenic chemicals or to the total mixture showed increased outgrowth whereas exposure to anti-androgens or paracetamol alone did not seem to affect outgrowth or differentiation of mammary glands of prepubertal female or male offspring. In another rat study, exposure to the estrogenic compound genistein similarly showed increased outgrowth of female mammary glands [Murrill et al. 1996]. Alteration of other parameters by estrogenic chemicals has also been reported in female mammary glands. Altered differentiation observed as a change in the lobule development relative to the number of TEBs has been observed for genistein and 17β-estradiol in rats and mice [Cabanes et al. 2004; Hilakivi-Clarke et al. 1997; Murrill et al. 1996]. The number of TEBs was not changed by exposure in the present study, and a change in differentiation may not be present. Estrogens have also been shown to affect prepubertal male mammary glands by increasing the branching and density of the gland [Mandrup et al. 2012; You et al. 2002], but in the current study, estrogens did not influence any of the examined parameters in males, including the density.

Previous studies have shown an increased prevalence of hypertrophy in adult male mammary glands exposed perinatally to phytoestrogens or genistein [Boberg et al. 2013; Delclos et al. 2001]. In a study by Boberg et al (2013, submitted), 80% of 6 months old males exposed to a mixture of phytoestrogens had hypertrophic mammary epithelium compared to 33% of controls. Similarly, Delclos et al. (2001) showed hypertrophic epithelium in mammary glands of 62% of 50 days old males exposed perinatally to genistein [Delclos et al. 2001]. In a study by Mandrup et al. (2013, submitted) hypertrophic epithelium was found in 88-90% of males PD90 exposed to ethinyl estradiol [Mandrup et al. 2013]. In the present study, the prevalence of hypertrophy in exposed males was 81%, however, more than half of control males displayed hypertrophic epithelium likely due to the higher age compared to the mentioned studies, and a change in the prevalence was not present. In the two previous studies in Sprague-Dawley rats, none of the 50 days old control males had hypertrophic epithelium [Delclos et al. 2001] and less than 30% of 6 months old control males

were hypertrophic [Boberg et al. 2013]. In a study with Wistar rats, 50% of controls had hypertrophic epithelium [Mandrup et al. 2013]. This difference in the prevalence of hypertrophy in control males may be due to the difference in strain or the age of the rat. The occurrence of hypertrophic epithelium appears to increase with age in controls and it may thus be preferable to evaluate male mammary glands for hypertrophy at an earlier age to detect a possible change in the susceptibility to develop hypertrophic mammary epithelium at an earlier age. Likewise, Lee et al. (2004) found age-related differences in sensitivity of mammary glands exposed to EDCs [Lee et al. 2004]. Thus, some changes may be naturally occurring with age and a difference in exposure-groups may not be observable after a certain age. Further studies on young post-pubertal and older male mammary glands may clarify this hypothesis.

Based on the present findings, mixed exposure to estrogenic chemicals appear to increase mammary gland growth and the susceptibility to mammary cancer in females exposed perinatally to estrogens. This may give rise to concern for the daughters of women exposed to estrogenic chemicals from various sources during pregnancy and in the breast feeding period. However, further studies on dose-levels are needed for risk assessment of the effects of perinatal estrogenic chemicals on the mammary glands, as no NOAEL was determined for changes in prepubertal female mammary glands.

No influence of anti-androgens on prepubertal mammary development

Anti-androgens alone did not affect the examined parameters in prepubertal or adult mammary glands in the present study. Accordingly, effects of anti-androgens on female and male mammary glands from prepubertal rodents have not been observed in previous studies [Jacobsen et al. 2012; Peters et al. 2011; Skarda 2003]. However, effects of anti-androgens have been described in rodents at puberty or older [Jacobsen et al. 2012; Lee et al. 2004; Peters et al. 2011; Saad et al. 2011]. In pubertal female mammary glands, perinatal exposure to anti-androgens has been found to increase branching and ductal hyperplasia [Saad et al. 2011]. In sexually mature rodents, anti-androgens have been described to increase branching [Peters et al. 2011] and increase the number of TEBs [Saad et al. 2011] in females. In adult males, anti-androgens were found to have feminising effects [Jacobsen et al. 2012] or to increase atrophy and degeneration of the mammary gland epithelium

[Lee et al. 2004]. However, histological evaluation of adult male mammary glands in the present study did not show effects of feminisation. This may be explained by different dose-levels of the anti-androgens studied with relatively high exposure levels applied in the study by Jacobsen et al. (2012).

Androgens have been described to be responsible for the male-like lobuloalveolar development of the male mammary glands [Sourla et al. 1998], but this morphology is only apparent in adulthood [Ahren and Etienne 1957; Cardy 1991; Sourla et al. 1998]. Thus the effects of anti-androgens on mammary glands may not be observable morphologically in males before a lobuloalveolar pattern can be expected and effects of anti-androgens are not observed morphologically in prepubertal male mammary glands.

The findings reported by Jacobsen et al (2012) suggests that early exposure to anti-androgens in male offspring can lead to changes in the mammary glands influencing the development of mammary glands later in life after sexual maturation, although the exposure is not continuing to adulthood. Saad et al. showed changes in the gene expression of estrogen receptors (ERs) and androgen receptors (ARs) in prepubertal and adult mammary glands of female Wistar rats exposed perinatally to the estrogenic compound genistein or the anti-androgenic compound vinclozolin [Saad et al. 2011]. The presence of ARs have been shown to be of importance for prepubertal, pubertal and adult mammary gland development in female mice [Yeh et al. 2003]. Changes in the levels of ARs and ERs before sexual maturation may thus influence mammary gland development in adult females and males. Further histological evaluation or gene expression of adult mammary glands of offspring exposed perinatally to EDCs may help to confirm or dismiss this hypothesis.

Mixture considerations

The combined exposure to estrogens and anti-androgens affected growth in female mammary glands to the same extent as the estrogens alone. Thus, the effect in the TotalMix-450 group is considered to be due to the estrogenic compounds present in the mixture, and the anti-androgenic compounds did not appear to influence this effect, i.e. no combination effects could be detected. Saad et al (2011) found an increased ductal proliferation and branching of the female mammary glands of female offspring exposed concomitantly to an estrogenic and an anti-androgenic chemical. However, this increase was similar to the changes seen in females exposed to the single

compounds genistein and vinclozolin [Saad et al. 2011]. This is in accordance with the findings in the present study, in which the increased outgrowth of the female mammary gland was comparable for estrogenic chemicals alone and for mixed exposure to estrogenic and anti-androgenic chemicals.

Histological examination of female mammary glands in 1-year old offspring showed an increased prevalence in the number of females showing lobular development with dilated and secretion filled alveoli and ducts in the TotalMix-450 group. Such changes may be related to high levels of prolactin [Neville et al. 2002]. Female rats are known to develop spontaneous pituitary tumours with age [Poteracki and Walsh 1998] and such changes in the mammary glands may be associated with the development of prolactin producing pituitary tumours. The changes observed in the TotalMix-450 group may indicate an increase in the prevalence of animals with signs of prolactin producing pituitary tumours in the mixture group. This may be attributed to estrogens and anti-androgens jointly.

Conclusion

Perinatal exposure to estrogenic chemicals increased outgrowth in mammary glands of prepubertal female offspring. The effect on outgrowth of estrogenic chemicals was retrieved in the TotalMix. In contrast, anti-androgenic effects on mammary gland morphology were not present in prepubertal mammary glands; however, changes may be observed in sexually mature male mammary glands. Histological examination of 13 months old females exposed to TotalMix-450 suggested an increased prevalence of pituitary tumours in this exposure-group, but this is yet to be confirmed. No other changes in adult mammary glands were found, and this may be explained by the age at examination. Changes observed around PD50 may not be detectable in 1-year old rats, if such changes are naturally occurring with age.

The present study showed estrogenic effects on mammary glands, but no NOAEL was determined for changes in prepubertal female mammary glands. Further studies on dose-levels are needed for risk assessment of the effects of perinatal estrogenic chemicals on mammary glands.

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Appendix 1

Historical control data in whole mounts – differences in strains and sub-strains

Appendix 1

Historical control data in whole mounts – differences in strains and sub-strains

Introduction

The number of TEBs in Wistar females in the study with ethinyl estradiol (mean number of TEBs=10) appeared smaller compared to the number of TEBs reported in Sprague-Dawley females in other studies (mean TEBs=30-43) [Cotroneo et al. 2002; Moral et al. 2008; Tan et al. 2004]. Control values for some parameters evaluated in whole mounts in mammary glands may not be comparable between studies due to differences in methods, strains or sub-strains. This appendix looks into the differences between strains and sub-strains (from another supplier) used in our studies. Control values of the same parameters evaluated in a Wistar rat strain from a different supplier and in Sprague-Dawley rats used in studies in our laboratory were investigated. Moreover, the present appendix looks into the differences in control values for the parameters area, distance to the lymph node, density and number of TEBs evaluated in whole mounts of mammary glands from the Wistar rat strain routinely used in our laboratory.

Methods

Animals

Data from historical controls from studies performed on female and male mammary gland whole mounts pup day 22 were collected. Most control values were from Wistar rats from a Danish supplier, Taconic. A single set of control values was from another sub-strain of Wistar rats from a Swedish supplier, Scanbur, and another set was from Sprague-Dawley rats, a different strain than Wistar. The mammary glands were processed in similar conditions (in the same laboratory and by the same technician), yet, one important step in the processing of the glands was changed in two studies with Wistar rats from Taconic. Clamps were used to squeeze mammary glands before fixation of the whole mounts in most studies (referred to as the clamp method) (Figure 1A). In two studies, mammary glands were pressed with two Plexiglas blocks of equal weight (referred to as the

weight method) (Figure 1B), resulting in less pressure than the clamp method before fixation of the whole mounts. Values for the area, distance to the lymph node, density and number of terminal end buds (TEBs) were assembled for control animals in tables 1 and 2. In the following, Wistar rats from Taconic will be referred to as Taconic rats and Wistar rats from Scanbur will be referred to as Scanbur rats.

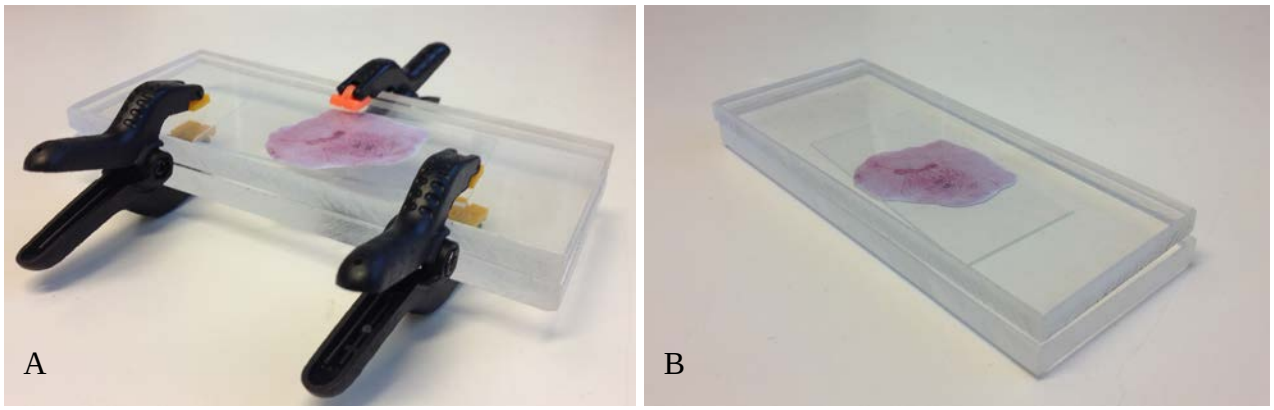

Figure 1. Different methods used for squeezing mammary glands before fixation in formalin. A, Mammary gland whole mount was placed between two plexiglass blocks and squeezed with clamps (referred to as the clamp method). B, Two plexiglass blocks were placed as weight on the mammary gland whole mount (referred to as the weight method).

Statistics

Statistical analysis was performed in SAS Enterprise Guide 4.3 statistical software (SAS Institute Inc, Cary, NC, USA) or GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set at $p=0.05$. A t-test was used to analyse pairwise comparisons of area, distance to the lymph node and density between sub-strains of Wistar rats (from Scanbur and Taconic), between the two strains (Wistar and Sprague-Dawley) and between two methods (weight- and clamp methods). For these pairwise analyses the number of TEBs was analysed with a non-parametric Mann Whitney test. For the evaluation of the differences within Wistar Taconic rats, control values for the area, distance to the lymph node and density were analysed with an ANOVA with a post hoc Tukey test to correct for multiple comparison of the difference in LS Means. The number of TEBs were analysed using a non-parametric Kruskal-Wallis test followed by a Dunn's

post hoc test to correct for multiple comparisons. The density was furthermore analysed in a non-parametric test similarly as the number of TEBs in all the comparisons. The area in Taconic rats was analysed for correlation with body weight with a two-tailed Pearson analysis.

Results and discussion

Control means for area, distance to the lymph node, density and number of TEBs for female and male mammary glands on PD22 are shown in table 1 and 2. Results from the statistical analysis are shown in table 3.

Table 1. Control data (mean ±SD) from mammary gland whole mounts from females PD22. Data included are from studies performed in the present thesis or historical control data from previous studies. Data from Wistar and Sprague-Dawley rats are included. Wistar rats were from either Taconic in Denmark or Scanbur in Sweden. Two different methods were used for the whole mount procedure, the clamp method or the weight method. Bw: body weight. Lnn: lymph nodes. P:paper. H: historical data.

Paper/ study	Strain – supplier	N (litter)	Bw	area	Distance to lnn	density	TEBs
Clamp method							
P1: Ethinyl estradiol	Wistar – Taconic	7	42±5	41±13	1.4±0.7	2.6±1.1	10±11
P3: Phyto-estrogens	Sprague-Dawley	14	50±6	93±22		2.9±1.8	
P4: Pesticides	Wistar – Taconic	11		49±14		3.5±1.0	25±19
P5a: Mix study	Wistar – Taconic	16	47±5	49±15	1.1±1.0	3.4±1.2	42±14
P5b: pre-study	Wistar – Taconic	10	55±6	79±16	1.2±1.1	2.5±1.1	6±9
H1: Historical data 1	Wistar – Taconic	17	44±5	64±19	1.0±1.1	3.9±0.6	8±7
H2: Historical data 2	Wistar – Scanbur	8	50±7	69±11	1.6±1.5	3.9±0.6	47±20
Weight method							
H3: Historical data 3	Wistar – Taconic	14	41±5	28±9	0.5±0.6		
H4: Historical data 4	Wistar – Taconic	14	46±6	30±11	0.4±0.6		

Table 2. Control data (mean ±SD) from mammary gland whole mounts from males PD21/22. Data included are from studies performed in the present thesis or historical control data from previous studies. Data from Wistar and Sprague-Dawley rats are included. Wistar rats were from either Taconic in Denmark or Scanbur in Sweden. Two different methods were used for the whole mount procedure, the clamp method or the weight method. Bw: body weight. Lnn: lymph nodes. P: paper. H: historical data.

Paper/ study	Strain	N (litter)	Bw	Area	Distance to lnn	Density	TEBs
Clamp method							
P1: Ethinyl estradiol	Wistar – Taconic	8	39±4	46±9	2.4±1.4	1.9±0.6	0.6±0.4
P4: Pesticides	Wistar – Taconic	14		42±12		2.9±1.1	6±5
P5a: Mix study	Wistar – Taconic	12	51±15	47±23	1.8±1.4	2.6±1.0	4±6
P5b: pre-study	Wistar – Taconic	10	56±6	67±18	1.7±1.8	2.6±0.5	1±1
H1: Historical data 1	Wistar – Taconic	17	46±5	49±16	2.6±2.0	2.7±1.1	0.5±1.1
H2: Historical data 2	Wistar – Scanbur	8	51±7	59±22	3.1±1.8	4.3±0.7	31±12
Weight method							
H3: Historical data 3	Wistar – Taconic	17	44±4	21±10	1.1±0.9		
H4: Historical data 4	Wistar – Taconic	10	47±6	28±10	0.6±0.3		

Table 3. Statistically significant differences found in control values from evaluation of whole mounts of female and male mammary glands. P-numbers refer to paper numbers and H-numbers refer to historic data numbers from tables 1 and 2.

Groups compared	Gender	Differences found
Strain differences: Sprague-Dawley vs. Wistar Taconic	Females	Area: Sprague-Dawley > Taconic
Sub-strain differences: Scanbur vs. Taconic Wistar rats	Females	Terminal end buds: Scanbur > Taconic
	Males	Density: Scanbur > Taconic Terminal end buds: Scanbur > Taconic
Differences within a Wistar sub-strain: Taconic Wistar rats	Females	Area: P5b > P1, P4 and P5a; H1 > P1 Density: H1 > P5b Terminal end buds: P5a > P1, P5b, H1
	Males	Area: P5b > P4, P5a.
Differences in methods: Weight vs. clamps	Females	Area: clamp s> weight Distance to the lymph nodes: clamps > weight
	Males	Area: clamps > weight Distance to the lymph nodes: clamps > weight

Mammary glands of Sprague-Dawley rats were only studied in females. The area of Sprague-Dawley rats was statistically significantly larger compared to mammary glands of Taconic rats ($p<0.0001$). The body weight of Sprague-Dawley females was comparable to Taconic rats. Similarly, the mean density of Sprague-Dawley rats was within the range of mean densities in Taconic rats. The distance to the lymph nodes and the number of TEBs was not assessed for Sprague-Dawley rats and a difference between strains could not be investigated for that parameter.

Scanbur males differed from Taconic males. Males from Scanbur had higher densities compared to Taconic males ($p<0.001$). Moreover, Scanbur rats had more TEBs than Taconic rats in females as well as males ($p<0.01$ in females and $p<0.0001$ in males). No differences were observed between Scanbur and Taconic females, except for the difference in the number of TEBs.

Taconic rats had equal means for the distance to the lymph node in females and males and the number of terminal end buds in males. Additionally, the densities were equal in means; however, females in the pre-study in paper 5 had lower mean density compared to controls from the historical study 1 (H1). The number of TEBs in females was higher for the study in paper 5 compared to other Taconic females. Statistically significant difference in the area in female and male mammary glands was observed in Taconic rats. A Pearson analysis showed more marked correlation between area and body weight in Taconic females compared to males ($r^2= 0.4$ in females and $r^2= 0.1$ in males) and a variation in the area may reflect a variation in body weight in females (Figure 2). Accordingly, rats from the pre-study in paper 5 were generally larger compared to other Taconic rats (55g compared to 42-47g in other Taconic rats) and mammary glands from these animals were in general larger than other Taconic mammary glands (mean area=79 compared to 41-49 in female Taconic rats with significantly smaller mammary glands).

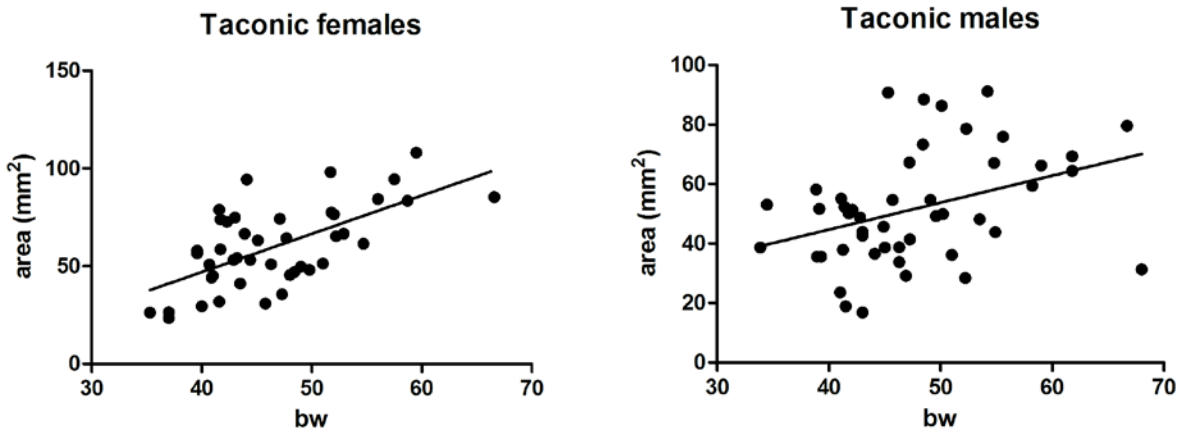

Figure 2. Correlation between area and body weight (bw) in Wistar rats from Taconic.

The two different methods used showed effects on the mammary outgrowth. The area ($p<0.0001$) and the distance to the lymph node ($p<0.01$) were increased in mammary glands squeezed by clamps. The density and the number of TEBs were not determined for the studies using the weight method.

Discussion

Rat strains, sub-strains and rats from the same sub-strain can differ substantially in mammary gland size, density and number of TEBs in controls, although parameters are measured at the same age, animals are housed under the same conditions and glands are processed in the same laboratory.

Strain differences were apparent in the area. Sprague-Dawley (S-D) mammary glands were larger prepubertally than Wistar Taconic mammary glands in our studies, whereas female Long Evans (LE) rats have been reported to have mammary glands about the size of our Wistar rats PND 21 (area in female LE rats: 25.1±2.1; mean±SEM) [Hovey et al. 2010]. The number of TEBs was not possible to count in the whole mounts from Sprague-Dawley rats in the present studies, but several studies have reported a larger number of TEBs in female Sprague-Dawley rats PND21 (TEBs in S-D rats: 30±5 - 43±5; mean±SEM) compared to our Wistar Taconic mammary glands [Cotroneo et al. 2002; Moral et al. 2008; Tan et al. 2004]. Female Long Evans rats were reported to have a

number of TEBs closer to the number reported in Sprague-Dawley females than in Wistar females from our studies, although the mammary gland area of Long Evans rats appeared to be similar to our female Wistar rats (TEBs in LE rats: 48.3±7.8; mean±SEM) [Hovey et al. 2010]. It appears that mammary glands have strain-specific morphologic features.

Differences were also observable within the same strain, between sub-strains, i.e. in Wistar rats purchased from different suppliers. Control values on outgrowth parameters evaluated in whole mounts from Scanbur rats were not significantly different from Taconic rats. However, control data revealed differences between the two sub-strains of Wistar rats for the number of TEBs in females and males and the density in males. These parameters in Scanbur rats were significantly higher compared to Taconic rats. Thus, Scanbur rats had more developed mammary glands compared to Taconic rats, although the glands were of similar sizes.

Within the same sub-strain, differences were also observed in mean control values between different studies. The distance to the lymph node and the number of TEBs for each gender and the density in males were found to be similar between studies for prepubertal Wistar Taconic rats. Area correlated with body weight and the gland area in rats from one study was generally larger than in other Taconic rats, likely due to the larger size of the animals. Moreover, the number of TEBs varied largely between the study in paper 5 and the other studies. This may be due to the different persons counting TEBs, as this procedure is somewhat subjective. Especially females have many TEBs and the number of TEBs may increase substantially if the borders of zone C are assessed differently or if the criteria used for defining a TEB are different.

Moreover, differences may also be seen between different studies using different techniques in the processing of mammary gland whole mounts. Statistically significant differences in the area and the distance to the lymph node were observed comparing the clamp method and the weight method. The area and distance to the lymph node measured in historical controls (from recent, unpublished studies) using the weight method were smaller than the areas and distances to the lymph node observed in other studies on Taconic rats using the clamp method. These differences may indicate a large variation in the size of female and male mammary glands in Wistar rats from Taconic. However, this could also be explained by the novel technique used with less pressure (weight method) spreading the mammary glands on the glass slides to a lesser extent compared to the clamp method.

Overall, area varied greatly from study to study and between Wistar and Sprague-Dawley strains. In contrast, the distance to the lymph node was only affected by the processing method used in the present studies, but was not different within Wistar Taconic rats or between Wistar sub-strains. Thus, less variation between studies was observed in the distance to the lymph node compared to the area. The number of TEBs was comparable for most Taconic rats; however, it appears that technical differences lead to a high number in a single study. Also sub-strain differences in the number of TEBs were observed. Further studies are needed to investigate the strain differences in the number of TEBs, although the literature suggests a higher number of TEBs in Sprague-Dawley rats compared to the Wistar rats studied in the present studies. Density was comparable for all strains and sub-strains except for Scanbur males, in which densities were higher compared to Taconic males.

Conclusion

Some parameters evaluated in whole mounts in mammary glands are not comparable between studies due to differences in strains, sub-strains or methods. Even within the same sub-strain, variation is seen in control means from study to study.

In Sprague-Dawley rats, only females and only the density and area were assessed, and the area of mammary glands was larger compared to Wistar rats. Further investigation of other parameters in Sprague-Dawley and other strains is needed to elaborate on strain differences. Differences in morphologic parameters in mammary glands in Wistar sub-strains were also found. Wistar rats from Scanbur had more developed mammary glands compared to Wistar rats from Taconic. However, outgrowth was similar between sub-strains.

Within the same sub-strain of Wistar rats differences were apparent in parameters like area, density and number of TEBs. Differences in area may be related to the different sizes of the animals with larger gland area in larger animals, especially in females. Another outgrowth parameter, the distance to the lymph node, appeared to be more consistent within the Wistar sub-strain. This parameter was, however, affected by the amount of pressure used for squeezing the gland.

Several parameters evaluated in whole mounts of prepubertal mammary glands vary greatly between strains, sub-strains and methods used in different studies and comparison of crude means between studies of such endpoints should be avoided.

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Appendix 2

Study report presenting evaluation of female external genital malformations

Appendix 2

Study report presenting evaluation of female external genital malformations

Introduction

The objective was to investigate whether the development of female external genitals is sensitive to exposure to endocrine disrupting chemicals (EDCs). The goals were (i) to define a sensitive parameter in female external genitals and (ii) to evaluate if female external genital malformation is a sensitive endpoint to environmentally relevant EDCs.

Based on findings reported in studies investigating the effects of EDCs on female external genital malformations [Flaws et al. 1997; Gray et al. 1997; Gray and Ostby 1995; Sawaki et al. 2003a; Sawaki et al. 2003b; Wolf et al. 2002; Yamasaki et al. 2005], four different methods for evaluation of the genitals were evaluated in the present studies in female offspring after exposure to a potent estrogenic chemical, ethinyl estradiol. The urethral slit length was assessed to be the most reliable measurement sensitive to ethinyl estradiol and was then studied in offspring exposed perinatally to environmentally relevant EDCs.

Materials and methods

Animals, chemicals and study design

Animals, environmental conditions and diet are described in papers 2 and 5. Details on these parameters are similar for all studies performed. A study with a potent estrogenic compound, ethinyl estradiol, was performed as a positive control study. Details on study design of the positive control study are described in paper 2. Furthermore, studies with the environmentally relevant EDCs bisphenol A (estrogenic), epoxiconazole (anti-estrogenic), and mixtures with either estrogenic (Emix), anti-androgenic (Amix) or EDCs with dissimilar modes of action (TotalMix) were performed. The study design on the mixture studies is described in paper 5. The study designs of the studies on bisphenol A and epoxiconazole are similar to the studies described in paper 2 and 5. Chemicals, doses, number of litters, ages for the evaluation of female external genital

malformations and papers describing the study in details are shown in Table 1. The day of expected delivery was defined as pup day (PD) 0.

Table 1. Studies performed for evaluation of the female external genital malformations. Chemicals, doses, ages evaluated for genital malformations and papers where the study is described are shown.

Chemical	Doses	n	Ages evaluated	Paper
Ethinyl estradiol	5, 15 and 50 µg/kg	10	PD 22, 50 and 90	2
Bisphenol A	0.025, 0.25, 5, 50 mg/kg	22	PD22 and 90	NA
Epoxiconazole	15, 30 mg/kg	18	PD22 and 90	NA
Paracetamol	350x	16-20	PD22, 50, and 400	5
Emix	200, 450x	16-20	PD22, 50, and 400	5
Amix	200, 450x	16-20	PD22, 50, and 400	5
TotalMix	100, 200, 450x	16-20	PD22, 50, and 400	5

n: number of litters. Paper: study design described in one of the five papers of this PhD. NA: not applicable – not described in a paper. x: fold high human exposure.

Evaluation of a sensitive endpoint

Several measurements were performed on female external genitals at PD22, 50 and 90 in the positive control study. For each age, the urethral slit length, the urethral-vaginal distance, the relative length of the urethral slit compared to the length of the genital papilla and an evaluation of the presence or absence of a clefted genital papilla were evaluated. All measurements were performed in a stereomicroscope with a scale. The measurement of the urethral slit length was performed as described in paper 2. The urethral-vaginal distance was measured as the distance between the bottom of the cleft and the vaginal opening. To calculate the relative urethral slit length ($relative\ urethral\ slit\ length = \frac{urethral\ slit\ length}{length\ of\ genital\ papilla} * 100$), the length of the genital papilla was calculated as the sum of the urethral slit length and the urethral-vaginal distance. The presence or absence of a clefted genital papilla was evaluated by determining a threshold for the normal cleft (urethral slit length in controls). The sides of the urethral slit were separated manually and the

measurements were performed with triple repetition to avoid false negative results from the sides of the urethral slit sticking together.

Evaluation of endocrine disrupting chemicals

The urethral slit length was measured in female offspring exposed perinatally to single compounds with endocrine disrupting activity – epoxiconazole, bisphenol A or paracetamol – or mixtures of estrogenic chemicals (Emix), anti-androgenic chemicals (Amix), or a mixture of EDCs with dissimilar modes of action (TotalMix). Female external genitals were evaluated at PD22, 50 and in 13 months old (PD400) offspring.

Statistics

The statistical programmes SAS Enterprise Guide 4.3 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used for the statistical analyses. Quantitative data (urethral slit length, relative urethral slit length, urethral-vaginal distance and genital papilla length) were analysed using an analysis of variance (ANOVA) with body weight as a covariate and a Dunnett's post-hoc test. If several pups per litter were evaluated, the litter was included as a random factor. The presence or absence of a clefted genital papilla was analysed with a Fisher's exact test.

Results

Evaluation of a sensitive endpoint

In the study with ethinyl estradiol, no statistically significant changes were seen for the urethral slit length or the relative urethral slit length on PD22 and PD50, nor for the qualitative evaluation of the presence of a clefted papilla on PD22 and PD90 (Table 2). The urethral slit length and the relative urethral slit length were statistically significantly increased on PD90 at 50 µg/kg. The urethral-vaginal distance was decreased in all ages evaluated. The prevalence of females with a clefted

genital papilla was increased in the highest dose-group PD50. Results for the urethral slit length in the positive control study are also described in paper 2.

Table 2. Results of measurements in female external genitals after perinatal exposure to ethinyl estradiol. Arrows: statistically significant increase or decrease. Parentheses specifies at which dose level a statistically significant change was observed.

	PD22	PD50	PD90
Urethral slit length	-	-	↑ (50 µg/kg)
Length of genital papilla	↓ (50 µg/kg)	↓ (15 µg/kg)	↓ (50 µg/kg)
Realtive urethral slit length	-	-	↑ (50 µg/kg)
Urethral-vaginal distance	↓ (50 µg/kg)	↓ (15 µg/kg)	↓ (50 µg/kg)
Clefted genital papilla	-	↑ (50 µg/kg)	-

The distances to the vaginal opening were difficult to assess. Thus, the urethral slit length was measured in studies on EDCs.

Evaluation of endocrine disrupting chemicals

No statistically significant effects were found in the urethral slit length in offspring exposed to environmentally relevant EDCs *in utero* and during the lactation period (data not shown).

Discussion

Evaluation of female external genital malformations

Measurement of the parameters in the female external genitals was somewhat complicated. The parts of the papilla that form the urethral slit can stick together and a urethral slit may not be visible. To evaluate the urethral slit length and other parameters measured for the evaluation of genital

malformations, these parts must be separated by digital manipulation. If such manipulation is not performed, false negative results may follow showing small urethral slits, although a deep urethral slit is present. Moreover, digital manipulation of the genital papilla can provoke excretion of secretory material (probably from the glandulae preputiales) at the tip of the genital papilla. The urethral slit length may appear longer due to this secretion and may lead to false positive results. Secretory material should be wiped off before measurement. It is thus imperative to be aware of these difficulties to secure proper measurement of the urethral slit length.

A decrease in the length of the papilla was accompanied by a decrease in the urethral-vaginal distance. The boundary of the vaginal opening is difficult to decide and the distance to the vaginal opening is difficult to assess. This measurement is very dependent on the person measuring. To assess these parameters, the person must be trained and the same person must perform the measurements throughout the study. Effects on the distance to the vaginal opening may be a chance finding. Thus, this method was not used in following studies although effects were observed at all ages. The relative urethral slit length was also dependent on the length of the genital papilla and was accordingly not used in the following studies either.

The urethral slit length was not dependent on the length of the genital papilla and was the most precise measurement and thus the most credible parameter reflecting the genital malformations of the urethral slit. The urethral slit length was used to determine the presence or absence of a clefted genital papilla and both these parameters were evaluated in the following studies on EDCs.

Sensitivity of the urethral slit length to EDCs

The urethral slit length was increased in 90 days-old female offspring exposed perinatally to 50 µg/kg ethinyl estradiol. The parameter was not changed in 22 or 50 days old offspring compared to controls. EDCs with estrogenic, anti-androgenic or mixtures of EDCs with dissimilar modes of action did not show effects on the urethral slit length.

Conclusion

The urethral slit length was sensitive to ethinyl estradiol and was the most reliable measurement used for evaluation of female genital malformations. In the present studies, the urethral slit length did not appear to be sensitive to environmentally relevant EDCs.

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