Effects of azole fungicides on the function of sex and thyroid hormones

Kjærstad, Mia Birkhøj; Andersen, Helle Raun; Taxvig, Camilla; Hass, Ulla; Petersen, Marta Axelstad; Metzdorff, Stine Broeng; Vinggaard, Anne

Publication date:
2007

Document Version
Publisher's PDF, also known as Version of record

DANISH MINISTRY
OF THE ENVIRONMENT
Environmental
Protection Agency

Effects of azole fungicides on the function of sex and thyroid hormones

Mia Birkhøj Kjærstad and Helle Raun Andersen
University of Southern Denmark, Institute of Public Health,
Research Unit of Environmental Medicine

Camilla Taxvig, Ulla Hass, Marta Axelstad, Stine Metzdorff and Anne Marie Vinggaard
Technical University of Denmark, the National Food Institute,
Department of Toxicology and Risk Assessment

Pesticides Research ·**No. 111** 2007

The Danish Environmental Protection Agency will, when opportunity offers, publish reports and contributions relating to environmental research and development projects financed via the Danish EPA.

Please note that publication does not signify that the contents of the reports necessarily reflect the views of the Danish EPA.

The reports are, however, published because the Danish EPA finds that the studies represent a valuable contribution to the debate on environmental policy in Denmark.

Contents

Preface

The research presented in this report on endocrine disruption of azole fungicides was carried out from October 2004 to December 2006.

The involved institutions were Environmental Medicine, Institute of Public Health, University of Southern Denmark (SDU) and The National Food Institute, Technical University of Denmark (DTU).

The research was financially supported by the Danish Environmental Protection Agency's Pesticide Research Programme. In this context the authors wish to acknowledge the Danish Environmental Protection Agency for funding the project.

The presented research was made possible with the outstanding contributions of laboratory technicians and keepers of whom we wish to thank:

Ranja Bjerring, University of Southern Denmark, Odense
Birgitte Møller Plesning, the National Food Institute, DTU
Heidi Letting, the National Food Institute, DTU
Morten Andreasen, the National Food Institute, DTU
Anne Ørngren & Co. from the animal facilities, The National Food Institute, DTU
Lillian Sztuk, the National Food Institute, DTU

A steering committee for the project was established and chaired by Jørn Kirkegaard, the Danish Environmental Protection Agency (EPA):

Annika Boye Petersen, Danish EPA (October 2004 – September 2005)
Susanne Hougaard, Danish EPA (September 2005 – December 2006)
Henrik Leffers, Department of Growth and Reproduction, Rigshospitalet, Copenhagen University Hospital
Mari-Ann Flyvholm, National Research Centre for the Working Environment, Denmark
Karin Sørig Hougaard, National Research Centre for the Working Environment, Denmark (From May 2005-end)
Lise Grupe Larsen, Hospital of Storstroem County, Næstved
Jann Hau, Department of Experimental Medicine, University of Copenhagen
Sven Edelfors, Department of Pharmacology, University of Copenhagen
Lisbeth Knudsen, Institute of Public Health, University of Copenhagen

We would like to thank the members of the steering committee for their involvement through out the project period. Especially we want to thank the referees: Susanne Hougaard, Henrik Leffers, Karin Sørig Hougaard, and Sven Edelfors for their constructive comments during the writing process of this report.

Sammenfatning

Baggrund
Der er en stigende bekymring for, at selv lave eksponeringer for pesticider under udviklingen af reproduktionsorganer og nervesystem, kan medføre vedvarende skader på disse organsystemer. Fostre (og hermed gravide kvinder) og børn antages derfor at være særlige sårbare grupper i forhold til pesticideksponering.

Det overordnede formål med dette projekt var at undersøge effekter af tre hyppigt anvendte triazolfungicider, tebuconazol, propiconazol og epoxiconazol, på det endokrine system med speciel fokus på køns- og skjoldbruskkirtelhormoner. Desuden ønskede vi at sammenligne resultaterne med vores tidligere fund for imidazolfungicidet prochloraz, som viste sig at have hormonforstyrrende egenskaber i en række *in vitro* og *in vivo* undersøgelser. Hvis de tre triazolforbindelser har tilsvarende effekter som prochloraz tyder det på at disse effekter er generelle for azolfungicider.

Azol-fungicider anvendes i store mængder til bekæmpelse af svampesygdomme i kornafgrøder og i mindre omfang i prydplante-, grøntsags- og frugtproduktionen. I 2005 blev der således i Danmark samlet anvendt over 100 t aktivstof af disse midler. Da de er relativt fedtopløselige og let optages over mave-tarmkanalen kan befolkningen blive udsat for stofferne, hvis de findes som rester i fødevarerne. Aktuelt kontrolleres restindholdet af stofferne i frugt og grønt, men kun få azol-fungicider måles i korn og kornprodukter. Bl.a. måles det mest anvendte azol-fungicid, epoxiconazol, ikke, hvorfor det ikke er muligt at foretage en samlet vurdering af befolkningens eksponering gennem kosten. Generelt har azol-fungiciderne en lav akut giftighed, men der vides meget lidt om deres mulige sundhedsrisici ved lave kroniske eksponeringer. Vi har for nylig vist at azol-fungicidet, prochloraz, kan interagere med flere forskellige receptorer, heriblandt østrogenreceptoren (ER), androgenreceptoren (AR) og arylhydrocarbon receptoren (AhR) i cellebaserede *in vitro* assays. Desuden virkede prochloraz anti-androgent i voksne hanrotter og påvirkede serumkoncentrationerne af skjoldbruskkirtelhormoner. Ved eksponering i drægtighedsperioden og under diegivningen medførte stoffet en virilisering af hun-ungerne og en feminisering af han-ungerne.

AhR er en nuklear transkriptionsfaktor, som efter binding af ligand, medfører øget genekspression af AhR responsive gener så som CYP1-genfamilien. Den klassiske AhR ligand er 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) men også andre planare aromatiske polycycliske molekyler kan aktivere receptoren. Der er endnu ikke identificeret en naturlig endogen ligand. AhR medieret induktion af CYP1A1 og 1A2 vil blandt andet føre til øget omdannelse af østradiol til 2- og 4-hydroxyøstradiol og kan derfor påvirke organismens hormonkoncentration.

I dette projekt har vi undersøgt om de tre mest anvendte triazol-forbindelser i Danmark: epoxiconazol, propiconazol og tebuconazol, besidder de samme egenskaber som imidazolfungicidet prochloraz med hensyn til at reagere med ER, AR og AhR og til at påvirke steroidhormonsyntesen og aktiviteten af

enzymet aromatase, som konverterer testosteron til østrogen. Effekterne er undersøgt i etablerede *in vitro* assays. Desuden har vi etableret et bioassay (T-screen) til at undersøge binding til og aktivering af receptoren for skjoldbruskkirtlens hormoner, thyroxin (T4) og tri-iodothyronin (T3), og anvendt dette assay til at undersøge om prochloraz, epoxiconazol, propiconazol og tebuconazol kan virke som T3/T4 agonister eller antagonister.

Dernæst har vi undersøgt anti-androgene virkninger for tebuconazol og propiconazol *in vivo* i unge voksne hanrotter i Hersberger testen. Endelig er effekter på fostres udvikling undersøgt for epoxiconazol og tebuconazol ved at dosere hunrotter i drægtigheds- og diegivningsperioden og undersøge fostre og unger.

Årsagen til at propiconazol blev erstattet med epoxiconazol i undersøgelserne vedrørende effekter på fostrets udvikling, er at vi efter projektets start fik oplysninger om at forbruget af epoxiconazol var steget markant i 2004 og nu var det mest anvendte azolfungicid i dansk landbrug.

In vitro effekter

De tre azolfungicider, propiconazol, tebuconazol og epoxiconazol kunne alle, i lighed med prochloraz, virke hormonforstyrrende via flere forskellige mekanismer, selvom deres potens i nogle assays var lavere end for prochloraz.

De tre triazoler var således mindre potente ER antagonister (prochloraz > tebuconazole ≈ epoxiconazole ≈ propiconazole) og AhR agonister (prochloraz >>> tebuconazole ≈ epoxiconazole ≈ propiconazole) end prochloraz mens deres affinitet for AR svarede til prochloraz. Ligesom prochloraz hæmmede triazolerne enzymet aromatase dog igen med mindre potens end prochloraz (prochloraz >epoxiconazole ≈ tebuconazole > propiconazole).

Evnen til at påvirke syntesen af steroidhormoner blev undersøgt i en human binyrebark-cancercelle H295R. I lighed med prochloraz øgede alle tre triazolfungicider produktionen af progesteron mens produktionen af testosteron og østrogen var reduceret. Dette indikerer en hæmning af enzymer som er involveret i omdannelsen af progesteron til testosteron.

Ingen overbevisende effekter blev observeret med hensyn til interaktion med receptoren for skjoldbruskkirtlens hormoner for hverken prochloraz eller nogle af triazolerne.

Anti-androgene virkninger *in vivo*

I Hershberger testen, hvor anti-androgene effekter undersøges *in vivo*, havde hverken propiconazol eller tebuconazol i doserne 50, 100 eller 150 mg/kg lgv/dag nogen effekt på vægten af reproduktionsorganer eller på hormonniveauer, undtagen for den højeste dosis af propiconazol, hvor der var en signifikant stigning i serumkoncentrationen af follikel stimulerende hormon (FSH). Den eneste signifikante effekt på genekspressionen var nedsat ekspression af ornithin decarboxylase (ODC) mRNA i ventrale prostata ved alle doser af propiconazol og ved den højeste dosis af tebuconazol. Dette gen reguleres af androgener, men også via andre mekanismer. Da der ikke sås andre anti-androgene effekter (nedsat vægt af androgenafhængige organer, nedsat ekspression af andre androgenregulerede gener eller en stigning i LH) er det sandsynligt, at den observerede effekt på ODC mRNA skyldes en mekanisme som ikke er reguleret af androgener.

Konklusionen er derfor, at ingen af de to triazol-fungicider, propiconazol eller tebuconazol, blokerer androgen receptoren *in vivo* i Hershberger testen ved doser på 150 mg/kg lgv/dag og derunder. Dette er i kontrast til prochloraz, som inducerede markante anti-androgene effekter i Hersberger assayet ved doser mellem 50 og 150 mg/kg lgv/dag.

Effekter på afkommet efter perinatal eksponering

Drægtige rotter blev doseret med epoxiconazol (15 eller 50 mg/kg lgv/dag) eller tebuconazol (50 eller 100 mg/kg lgv/dag). Nogle dyr fik udført planlagt kejsersnit på gestationsdag (GD) 21 for at undersøge effekter på fostrene. Andre dyr fødte normalt og afkommet blev undersøgt enten ved fødslen eller postnatal dag (PND) 13 eller 16 efter at havde været yderligere eksponeret gennem modermælken. Den højeste dosis af epoxiconazol medførte øget fosterdød og gav tydelige problemer med de almindelige fødsler. Derfor blev der kun født 2 kuld mens de resterende moderdyr i denne gruppe fik foretaget kejsersnit mellem GD23 og GD25 og blev inkluderet i gruppen som fik foretaget kejsersnit ved GD21. Tolkningen af resultaterne fra undersøgelser af unger fra højdosis epoxiconazol gruppen er derfor vanskelige og der kan ikke drages endelige konklusioner.

De nedenstående effekter blev observeret for moderdyr, fostre og afkom: Både epoxiconazol og tebuconazol øgede drægtighedslængden. Det har vi også tidligere set for prochloraz (30 mg/kg lgv/dag). Denne effekt skyldes sandsynligvis, at azol-fungiciderne inducerer et øget progesteron niveau hos mødrene.

Afstanden mellem anus og kønsorganerne (’anogenital distance’, AGD) var øget i hununger efter dosering af mødrene med den højeste dosis af tebuconazole, samt den laveste dosis af epoxiconazol. Dette indikerer en virilisering af hunungerne. For de samme to dosisgrupper var der ingen effekt på AGD i nyfødte hanunger. Efter dosering med den højeste dosis tebuconazol var testosteronkoncentrationen i hanfostrenes testikler nedsat, mens progesteron og 17α-hydroxyprogesteron var øget ved begge doser af tebuconazol. Tebuconazole medførte yderligere en signifikant stigning i antallet af brystvorter samt en tendens til nedsat plasmakoncentration af testosteron i hanungerne. Det overordnede billede er således at tebuconazol viriliserer hunungerne og feminiserer hanungerne i lighed med prochloraz.

Epoxiconazol har tilsyneladende en vækstfremmende effekt på hanungerne. En højere fødselsvægt af ungerne er sandsynligvis forårsaget af et markant forøget testosteronniveau i moderdyret, der kan tænkes at virke vækstfremmende på ungerne. Dette kan også forklare den umiddelbare tendens til øget AGD i hanfostre og støttes af en ikke signifikant tendens til øget vægt i androgen-afhængige organer i hanunger. Alt i alt tyder resultaterne på at epoxiconazol’ virkningsprofil på hanunger er forskellig fra prochloraz’ og tebuconazols’ virkningsmåde. Stoffet er markant fosterbeskadende og hvilke mekanismer der ligger til grund for denne effekt og om hormonforstyrrelser er en del af årsagen, kan ikke siges ud fra vores forsøg.

Konklusion

Overordnet viser resultaterne fra dette projekt, at azol-fungicider generelt har samme virkningsprofil *in vitro*, men at virkningsprofilen *in vivo* er forskellig fra stof til stof. Resultaterne fra *in vitro* forsøgene viser at alle fire azoler har

potentialet til at virke hormonforstyrrende via en række forskellige mekanismer. Potensen af de tre triazoler som indgår i denne undersøgelse: epoxiconazol, propiconazol og tebuconazol var enten lig med eller lavere end imidazolfungicidet prochloraz' i de anvendte *in vitro* assays. Propiconazol og tebuconazol udviste ingen AR blokerende virkning *in vivo* i Hershberger assayet. Til trods for dette var både epoxiconazol og tebuconazol i stand til at medføre effekter på udviklingen af det reproduktive system i afkommet efter eksponering i livmoderen. Både epoxiconazol og tebuconazol inducerede virilisering af hunafkommet, mens tebuconazol også medførte feminisering af hanafkommet. Derudover virkede epoxiconazol generelt fosterbeskadende. Samlet set har de tre undersøgte azolfungicider haft det til fælles at de alle øger drægtighedslængden, de viriliserer hunungerne og de forøger progesteronniveauerne.

Disse effekter kan skyldes at flere virkningsmekanismer, som virker på samme tid, forstærker det samlede biologiske respons. Effekterne på steroidhormonsyntesen *in vitro*, udeblivelse af effekter i Hershberger assayet (hvor effekter på hormonsyntesen omgås ved at anvende kastrerede, testosteron supplementerede handyr) kombineret med effekterne på udviklingen af det reproduktive system efter perinatal eksponering tyder kraftigt på at en af hovedmekanismerne er forstyrrelse af nøgle-enzymer i steroidhormonsyntese.

De observerede virkninger fortjener videre undersøgelser med henblik på at afdække ved hvilke doser effekterne opstår og for at opnå flere oplysninger om hvilke mekanismer, der er involveret. Resultaterne fra projektet har givet ny viden om azol-fungiciders toksiske virkningsmekanismer og mulige sundhedsmæssige effekter på reproduktionen og det endokrine system og vil derfor kunne bidrage til en bedre risikovurdering for denne meget udbredte gruppe af svampemidler.

Summary

Background

There is a growing concern of permanent damage to the endocrine and nervous systems after exposure to even low levels of pesticides under development. Fetuses (i.e., pregnant women) and children are therefore considered as particularly vulnerable groups in relation to pesticide exposure.

The main objective of this project was to investigate the effects of three frequently used triazole fungicides, tebuconazole, propiconazole, and epoxiconazole on the endocrine system with special focus on sex and thyroid hormones. In addition, we wanted to compare the results with our previous findings for the imidazole fungicide, prochloraz, which was shown to possess endocrine disrupting effects in a number of *in vitro* and *in vivo* studies. If the triazole compounds possess similar effects as prochloraz these effects are likely to be general for the group of azole fungicides.

Azole fungicides are used in large amounts for control of fungi in grain production and to a lesser extent in the flower, vegetable and fruit production. Thus, in 2005 a total amount of 100 t of azole fungicides was used in Denmark. The fungicides are relatively fat-soluble and readily absorbed in the gastrointestinal tract. Therefore, the public is exposed to the fungicides when residues exist in food products. Residues of azole fungicides are monitored in vegetable and fruit, but only few azole fungicides are monitored in grain and grain products. For instance, the most widely used azole, epoxiconazole, is not monitored, making it impossible to do a complete dietary exposure assessment of the Danish population. In general, the azole fungicides have a low acute toxicity but the knowledge about their potential health risks at low chronic exposures is very limited. We have recently shown that the azole fungicide, prochloraz, can interact with several receptors, including the estrogen receptor (ER), androgen receptors (AR), and the aryl hydrocarbon receptor (AhR), in cell-based *in vitro* assays. Besides, prochloraz was anti-androgenic in adult male rats and affected the serum concentration of thyroid hormones. After exposure during pregnancy and lactation it caused feminization of male and virilization of female offspring.

The AhR is a nuclear transcription factor that, after binding of ligand, causes increased gene transcription of AhR responsive genes such as the CYP1 gene family. The classical AhR ligand is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) but also other planar aromatic polycyclic molecules activates the receptor. No natural endogenous ligand has yet been identified. AhR mediated induction of CYP1A1 and 1A2 will enhance the metabolism of estradiol to 2- and 4-hydroxy estradiol (Rifkind, 2006) and could therefore affect the hormone concentration in the organism.

In this project we have investigated whether the three actually most widely used triazole fungicides in Denmark: epoxiconazole, propiconazole, and tebuconazole, possess similar properties as the imidazole fungicide prochloraz regarding interactions with the ER, AR, and AhR, as well as effects on steroid hormone synthesis and the activity of the enzyme aromatase that converts testosterone to estrogen. The effects were investigated by established *in vitro*

assays. Furthermore, we have established a bioassay (T-screen) for detecting binding and activation of the thyroid receptor (TR) and used the assay to investigate the ability of prochloraz, epoxiconazole, propiconazole, and tebuconazole to be thyroid hormone agonists or antagonists. Additionally, we have investigated anti-androgenic effects of propiconazole and tebuconazole *in vivo* in young adult rats using the Hershberger assay. Finally, developmental effects of epoxiconazole and tebuconazole were investigated in rat offspring after exposure during pregnancy and lactation.

The reason why propiconazole was replaced by epoxiconazole in the developmental study was that we, after the project was initiated, were informed that the use of epoxiconazole had increased markedly during 2004 and now was the most widely used azole fungicide within Danish agricultural production.

In vitro effects

The three azole fungicides: propiconazole, tebuconazole, and epoxiconazole all had the potential, like prochloraz, to act as endocrine disruptors via several different mechanisms although the potencies were less than for prochloraz in some of the assays.

Hence, the three triazoles were less potent than prochloraz to act as ER antagonists (prochloraz > tebuconazole ≈ epoxiconazole ≈ propiconazole) and AhR agonists (prochloraz >>> tebuconazole ≈ epoxiconazole ≈ propiconazole) while the potency as AR antagonists was similar. Like prochloraz, the triazoles were aromatase inhibitors, although again, with lower potency than prochloraz (prochloraz >epoxiconazole ≈ tebuconazole > propiconazole).

The ability to interfere with steroid hormone biosynthesis was investigated in the human adrenocortical carcinoma cell line H295R. Like prochloraz, all three triazole fungicides caused enhanced production of progesterone and reduced production of testosterone and estradiol. This indicates that enzymes involved in the conversion of progesterone to testosterone are inhibited.

Convincing interactions with the TR were not found for either prochloraz or any of the triazole fungicides.

Anti-androgenic effects *in vivo*

In the Hershberger assay, used for investigating anti-androgenic effects *in vivo*, propiconazole and tebuconazole (50, 100, or 150 mg/kg bw/day) had no effect on neither reproductive organ weights or on hormone levels, except for the highest dose of propiconazole, for which there was a significant increase in the serum concentration of follicle stimulating hormone (FSH). The only significant effect of the azole fungicides on gene expression was a decrease in of expression of ornithine decarboxylase (ODC) mRNA in prostate at all doses of propiconazole and at the highest dose of tebuconazole. This gene is regulated by androgens but is also regulated via other pathways. Since none of the other anti-androgenic end points (weight of androgen dependent organs, expression of other androgen responsive genes or serum concentration of LH) were affected we believe that the effects observed on ODC mRNA expression are due to a non-androgen-regulated pathway.

In conclusion, none of the triazole fungicides, propiconazole or tebuconazole, had any androgen receptor blocking effect *in vivo* in the Hershberger test at

doses at or below 150 mg/kg bw/day. This is in contrast to prochloraz that induced anti-androgenic effects in this assay at doses between 50 and 150 mg/kg bw/day.

Effects on offspring after perinatal exposure

Pregnant rats were dosed with epoxiconazole (15 or 50 mg/kg bw/day) or tebuconazole (50 or 100 mg/kg bw/day). Some dams were chosen for caesarian section at gestational day 21 (GD21) to evaluate effects on sexual differentiation in the fetuses. Other dams delivered normally and the pups were examined at birth or at post natal day (PND) 13 or 16 after exposure during lactation. The highest dose of epoxiconazole caused marked fetal lethality and problems with normal delivery. Therefore only two litters were born normally and the remaining dams in this group had caesarian sections between GD23 and GD25 and they were included in the group having caesarian sections at GD 21. Interpretation of the results from examination of the offspring from the high dose epoxiconazole group is therefore hampered and no final conclusions can be drawn.

The following effects were observed for dams, fetuses, and offspring:
Both epoxiconazole and tebuconazole caused an increased gestational length as we have also previously seen for prochloraz (30 mg/kg bw/day). This effect is probably caused by a marked increase in plasma concentrations of progesterone in the mothers induced by both fungicides.

The anogenital distance (AGD) was increased in the female pups in the highest tebuconazole dose group and the lowest epoxiconazole dose group indicating a virilizing effect on the females. No effect on AGD was seen in the newborn male pups. The testosterone level in testis from the male fetuses was decreased by tebuconazole, while progesterone and 17α-hydroxyprogesterone levels were increased at both doses. Furthermore, tebuconazole caused a significant increase in the number of nipples in the male pups and a tendency towards decreased plasma testosterone concentration in male pups. Thus, the overall picture is that tebuconazole virilizes the females and feminizes the male pups.

Epoxiconazole had an apparent growth-promoting effect on the male offspring. A higher birth weight was seen which is probably caused by the enhanced testosterone level in the epoxiconazole-dosed mothers. The testosterone may have acted as a growth promoter and may have caused the increased AGD in the fetuses and the insignificant tendency towards an increased weight of androgen dependent reproductive organs in male pups. In summary, the results indicate that the profile of effects induced by epoxiconazol on male offspring is different from the effects induced by prochloraz and tebuconazol. Epoxiconazol has marked fetotoxic effects but the mechanisms behind and the potential role of endocrine disruption for these effects cannot be ruled out from this study.

Conclusion

Overall the results from this study indicate that azole fungicides in general have a similar profile of action *in vitro* but that the profile of action *in vivo* is different and varies from chemical to chemical. The four azole fungicides all have the potential to act via several modes of actions. The potencies of the three triazoles included in this study, epoxiconazole, propiconazole, and tebuconazole, were similar or less than for the imidazole fungicide, prochloraz, in the applied *in vitro* assays. Propiconazole and tebuconazole did

not possess androgen receptor blocking effect *in vivo* in the Hershberger test at doses at or below 150 mg/kg bw/day. Despite this, both epoxiconazole and tebuconazole were capable of inducing effects on reproductive development in the offspring after exposure *in utero*. Both epoxiconazole and tebuconazole virilized the female offspring, and tebuconazole also caused feminizing effects in male offspring. In addition epoxiconazol had marked fetotoxic properties. The common features for the tested azole fungicides are that they all increase gestational length, they virilize female pups, and they increase progesterone levels.

These effects are likely due to several different mechanisms operating simultaneously and thereby enhancing the integrated biological response. However, the effects on steroid hormone synthesis *in vitro*, the lack of effect in the Hershberger assay (where effects on steroid synthesis is omitted by using testosterone supplemented castrated male rats) combined with the effects on reproductive developmental after perinatal exposure strongly indicate that one of the main responsible mechanisms is disturbance of key-enzymes involved in the synthesis of steroid hormones.
The observed effects deserve further investigations in order to unveil the doses needed to induce the effects and to obtain more information on the mechanisms involved. The results from the project add new knowledge about the toxic mechanisms of azole fungicides and their potential for inducing health effects on reproduction and the endocrine system and will contribute to a better risk assessment of this widely used group of fungicides.

Abbreviations

17β-HSD:	17β-Hydroxysteroid Dehydrogenase
AGD:	Anogenital distance
AhR:	Arylhydrocarbon receptor
ALH:	Amplitude of lateral head displacement
AR:	Androgen receptor
ARE:	Androgen-responsive-element
ATCH:	Adrenocorticotropic hormone
CHO cells:	Chinese hamster ovary cells
Compl. C3:	Complement component 3
CYP:	Cytochrom P450
DCC:	Dextran charcoal treated fetal calf serum
DMSO:	Dimethyl sulfoxide
DREs:	Dioxin Responsive Elements
DTT:	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
EPA:	Environmental Protection Agency
ER:	Estrogen receptor
FBS:	Fetal bovine serum
FCS:	Fetal calf serum
FSH:	Follicle stimulating hormone
GD:	Gestational day
GH3:	A rat pituitary tumor cell line
H295R cells:	Human adrenocortical carcinoma cells
H4II3 cells:	Rat hepatoma cells
ICx:	Inhibitory concentration x %
IFMA:	Immuno fluorometric assay
IGF-1:	Insulin-like-growth factor 1
LABC:	Levator ani/bulbocavernosus muscles
LDH:	Lactate dehydrogenase
LH:	Luteinizing hormone
LOEC:	Lowest observed effect concentration
LOEL:	Lowest observed effect level
LUC:	Luciferase
MCF-7 cells:	a human breast cancer cell line
MOEC:	Maximum observed effect concentration
MMTV:	Mouse mammary tumor virus
ODC:	Ornithin decarboxylase
P450c17:	CYP17a1
P450scc:	Cytochrom P450-side-chain cleavage
PBP C3:	Prostate specific binding protein polypeptide C3
PBR:	Peripheral benzodiarepine receptor
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PEM:	Placenta and Embryos oncofetal gene
PND:	Postnatal day
RPE:	Relative proliferative effect
RT-PCR:	Reverse transcriptase PCR
sc:	Subcutane
ScarB1:	Scavenger receptor class B. member 1
StAR:	Steroidogenic acute regulatory protein

STR:	Straightness
T_3:	Triiodothyronine
T_4:	Thyroxine
TR:	Thyroid hormone receptor
TRPM-2:	Testosterone-repressed prostate message 2
TSH:	Thyroid stimulating hormone
VAP:	Average path velocity
VCL:	curvilinear velocity
VSL:	Straight line velocity

1 Introduction

1.1 Background

Exposures to pesticides during early development may cause permanent damages to the nervous and reproduction systems (Nielsen *et al.*, 2001). Therefore, children and fetuses (i.e. pregnant women) are particularly vulnerable towards pesticide exposure. This is due to the fact that the nervous and reproductive organ systems are not fully developed until late childhood and children have a higher dietary intake in relation to body weight than adults (Nielsen *et al.*, 2001). Besides, children often consume relatively much fruit and juice, which can be polluted with pesticides. Very little is known about the potential health risks at low chronic exposures but even low concentrations of endocrine disrupting pesticides in the diet might be of concern due to potential combination effects (Birkhoj *et al.*, 2004;Rajapakse *et al.*, 2002;Silva *et al.*, 2002).

Pesticides approved in Denmark have to be tested for adverse effects on reproduction and offspring by OECD guidelines TG 414 (Pre-natal Developmental Toxicity Study), 415 (One Generation Reproduction Toxicity Study), 416 (Two Generation Reproduction Toxicity Study), and 421 and 422 (Reproduction/Developmental Toxicity Screening Test) (http://www.oecd.org/home/). However, these test systems do not have an optimal design for revealing functional disruptions in the reproduction and nervous systems.

Recently we tested a range of commonly used pesticides for the ability to disrupt the endocrine system using a battery of *in vitro* tests (Andersen *et al.*, 2002; Long *et al.*, 2003). The pesticides were chosen due to their frequent use in Danish greenhouse horticultures (Andersen and Nielsen, 2001). Two thirds of the pesticides had endocrine disrupting properties and induced significant response in one or more of the *in vitro* tests. One of these pesticides was the imidazole fungicide prochloraz, which interacted in all the test systems. Prochloraz possessed anti-estrogenic and anti-androgenic effects, inhibited the activity of the estrogen synthesizing enzyme aromatase and activated the aryl hydrocarbon receptor (AhR). The AhR (or dioxin receptor) is an intracellular receptor that mediates the toxic responses of dioxin and related chemicals. Binding of a ligand to the receptor induce gene transcription of the CYP1A gene family and thereby the activity of some cytochrome P450 enzymes involved in the synthesis or metabolism of steroid hormones are increased (Rifkind, 2006).

In later studies, we found that prochloraz also induced anti-androgenic effects in rats *in vivo* (Vinggaard *et al.*, 2002) and feminized male offspring in rats after perinatal exposure (Vinggaard *et al.*, 2005a). The effects observed for prochloraz were comparable to effects reported for the fungicide vinclozolin (Gray, Jr. *et al.*, 1994;Gray, Jr. *et al.*, 1999a), which was banned because of its anti-androgenic effects. Furthermore, prochloraz decreased the concentration of thyroxin (T_4) and thyroid stimulating hormone (TSH) in serum of exposed

rats (Vinggaard *et al.*, 2005a) indicating interference with the thyroid function.

In Denmark the use of fungicides has been increasing with a total sale of 1625 t in 2001, 1744 t in 2003, and 2046 t in 2005. (Miljøstyrelsen, 2004 ;Miljøstyrelsen, 2006). The azole fungicides are used in large amounts in the control of fungi in grain and to a lesser extent in flower, vegetable and fruit production. The total amount of azole fungicides (as active ingredients) sold in Denmark in 2005 was over 100 t. Epoxiconazole alone accounts for 47 t (approximately half of the azole fungicides used) and prochloraz, propiconazole, and tebuconazole, accounts for 2, 31, and 26 t, respectively (Miljøstyrelsen, 2006).

The azole fungicides are relatively lipophilic and are absorbed through the gastrointestinal tract (Kampmann *et al.*, 1999). Therefore, the population can be exposed to azole fungicides through residues in the food. Residues of some azole fungicides are measured in fruit and vegetables but only few azoles are monitored in grain and grain products and for instance the most widely used azole fungicide in Denmark, epoxiconazole, is neither monitored in fruit, vegetables, grain nor grain products (Christensen *et al.*, 2006). Assessment of the total dietary exposure to azole fungicides of the human population is therefore hampered. Although the maximal residue limit was not exceeded for the individual azole compounds included in the 2005 survey, prochloraz or tebuconazol was detected in several samples of fruits and vegetables produced in Denmark or imported (Christensen *et al.*, 2006).

It is well known from the literature that several azole fungicides influence the activity of different cytochrome P450 enzymes and for instance inhibit the activity of aromatase (CYP19) which converts androgens to estrogens (Mason *et al.*, 1987; Sanderson *et al.*, 2002; Sanderson, 2006). Besides some *in vitro* studies on enzyme activities and our previous studies on prochloraz, only few other toxicological studies of these pesticides have been published despite of their massive use in agriculture and horticulture. In one study, exposure of pregnant rats to tebuconazole in doses around Lowest Observed Effect Level (LOEL) caused reduced weight of adult epididymis in male offspring and reduced adult uterus weight in female offspring. In addition, changes in behavior were observed (Moser *et al.*, 2001).

Prochloraz, propiconazole, and tebuconazole were assessed by the Danish Environmental Protection Agency (EPA) in 1998, 1996, and 1996, respectively (Pesticidkontoret, 1996a; Pesticidkontoret, 1996b; Pesticidkontoret, 1998). The toxicological assessments were mainly based on the studies reported by the manufactor in order to get the product approved by the authorities. At that time, none of the pesticides were assessed to be toxic to the reproduction system. Both prochloraz and tebuconazole were reported to disturb the thyroid function but this was not considered to be of importance to human health. Prochloraz was re-assessed in 2004 and based on new studies and a review of the endocrine disrupting effects of the compound from The Danish Institute for Food and Veterinary Research, prochloraz was categorized as toxic to the reproductive system and labeled with R61: “May cause harm to the unborn child” (Pesticidkontoret, 2004). Epoxiconazole was assessed in 2003 (Pesticidkontoret, 2003) and, classified as carcinogenic and toxic to the reproductive system in category 3 and labeled with R40, R62, and R63.

So far the research in endocrine disrupting mechanisms has primarily focused on disruption of sex hormones and related receptors but other mechanisms are also involved (Sharpe, 2006). Interference with steroid hormone biosynthesis (Sanderson, 2006) or the thyroid system (Boas *et al.*, 2006) are other important pathways of endocrine disruption.

The thyroid system is a central component of the endocrine system and thyroid hormones are essential for normal development and even small changes in thyroid homeostasis during critical periods of brain development may result in irreversible neurobiological damages and disruption of corresponding functions (Boas *et al.*, 2006; Porterfield, 1994; Porterfield, 2000). The fetus itself, do not produce thyroid hormone during the first half of the pregnancy and is consequently depending on the mothers hormones. Chemicals that can interfere with the thyroid hormone level of the mother are therefore suspected to be able to cause neurological damages to the child (Boas *et al.*, 2006; Porterfield, 1994). Ethylene thiourea, a metabolite of dithiocarbamate fungicides, is an example of a chemical that interferes with the thyroid system and causes thyroid hyperplasia and declined thyroid hormone levels (Lentza-Rizos, 1990). Different mechanisms of actions may be involved. The chemicals may bind directly to the thyroid hormone receptor (TR) or to the transport protein transthyretin or they may affect the release of TSH or the breakdown of thyroid hormones. For instance, the pesticides ioxynil, dicofol, and pentachlorphenol have been demonstrated to compete with triiodthyronine (T_3) for the binding to transthyretin while they do not bind to the TR (Ishihara *et al.*, 2003b; Ishihara *et al.*, 2003a).

Our research so far on prochloraz, combined with the few other studies of toxic effects of azole fungicides, indicate that these compounds have the potential to react through several mechanisms resulting in effects on various organ systems (Vinggaard *et al.*, 2005a). Our concern was that the endocrine disrupting properties demonstrated for prochloraz also applied to other azole fungicides, including triazole and other imidazole compounds. Hence, the aim of this study was to investigate if three other commonly used azole fungicides: epoxiconazole, propiconazole, and tebuconazole, possess comparable effects to those observed for prochloraz regarding interactions with the ER, AR and AhR *in vitro* as well as anti-androgenic and developmental effects *in vivo*. In addition, we wanted to investigate if prochloraz and the three other azole compounds interact with the TR *in vitro*.

Figure 1 illustrates the structure of the four azole fungicides. Prochloraz is an imidazole whereas the other three are triazoles. There is also a difference in the number of atomic chlorine attached to the phenyl rings and epoxiconazole differ from the others by containing atomic fluorine.

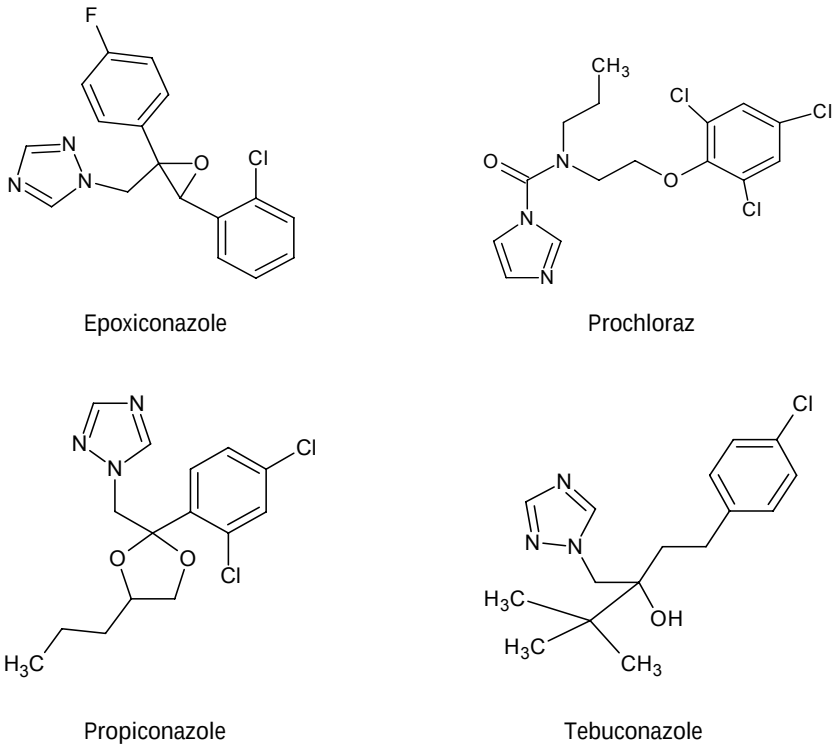


Figure 1 - Chemical structures of the four azole fungicides: epoxiconazole, prochloraz, propiconazole, and tebuconazole.

1.2 Project Objectives

The main objective of the project was to investigate the effects of three frequently used triazole fungicides on the endocrine system, including *in vitro* and *in vivo* examinations. Secondly, we wanted to compare the results with the imidazole fungicide, prochloraz, which previously has shown endocrine disrupting effects in a number of *in vitro* and *in vivo* studies.

1.3 Hypothesis and Strategy

The strategy of the project was to investigate:

1. Whether the triazole fungicides epoxiconazole, propiconazole, and tebuconazole possess similar properties as the imidazole fungicide prochloraz regarding interactions with the ER, AR, AhR, as well as effects on steroid hormone synthesis and the activity of the enzyme aromatase that converts testosterone to estrogen. The effects are investigated by established *in vitro* assays and the results are compared to the effects previously obtained for prochloraz. Differences in effects between the triazoles and prochloraz may indicate that the nature of the azole ring and/or the placement of the halogens in the phenyl rings, have an impact on the biological effect (Figure 1).

2. Whether prochloraz and the three triazoles interact with the TR. Tebuconazole and prochloraz have shown effects on the serum concentration of thyroid hormones in animal studies. The mechanism behind this effect is not known but one possibility is that the azole compounds may bind directly

to the TR. Chemicals with the ability to react both with the ER, AR and AhR might be capable to also react with other receptors or binding sites as reported for several other environmental toxicants (Laws *et al.*, 1995; Molina-Molina *et al.*, 2006; Sanderson, 2006). We therefore wanted to investigate the molecular interaction of the four azole fungicides with the thyroid receptor. A T-screen assay was established for this purpose.

3. Whether the three triazoles induce anti-androgenic effects in adult male rats (Hershberger assay). If so the effects will be compared with the effects previously obtained for prochloraz. Concentrations of pituitary and thyroid hormones and changes in expression of androgen responsive genes will be included to increase the sensitivity of the assay. Changes in gene expression, in the rat ventral prostate, are a valuable marker of anti-androgen action. Many genes contain an androgen-responsive-element (ARE) in the promoter region and their expression is directly influenced by the amount of androgens or anti-androgens available (Nellemann *et al.*, 2005).

4. Whether exposure to epoxiconazole or tebuconazole during pregnancy and lactation will cause effects on the offspring in rats. The fetus is the most sensitive stage of the organism towards endocrine disrupters. In an earlier study, feminization of male offspring was demonstrated after prochloraz exposure during pregnancy and lactation. The effects included feminization of male genitals, female-like areolas and feminized behavior. It is therefore highly relevant to investigate whether other azole fungicides induce similar endocrine disrupting effects during development.

2 Methods for testing endocrine disrupting effects of azole fungicides

2.1 *In vitro* testing

The first step in investigating endocrine disrupting properties of the azole fungicides was to screen the four compounds: epoxiconazole, prochloraz, propiconazole, and tebuconazole in a battery of cell assays for well-known mechanisms of endocrine disruption. The assay systems included the ability to induce proliferation in a human breast cancer cell line (MCF-7 cells) (estrogenic/anti-estrogenic effects), agonistic and antagonistic effects on the AR in transfected Chinese Hamster Ovary (CHO) cells, effects on activity of the estrogen synthesizing enzyme aromatase in the MCF-7 cell proliferation assay using testosterone as substrate, dioxin like effects estimated as activation of the Ah receptor in the CALUX assay, effects on thyroid function, by looking at the thyroid dependent cell proliferation of a rat pituitary tumour cell line (GH3 cells) in the T-Screen assay and finally effects on steroidogenesis in human adrenocortical carcinoma cells (H295R cells).

2.1.1 Estrogen/anti-estrogen testing – MCF-7 cells

The azole fungicides were tested for estrogenic and anti-estrogenic effects using a MCF-7 cell proliferation assay. The assay is based on a human breast cancer cell line. The cells depend on estrogen for growth. Proliferation of the cells is therefore an indication of the presence of an estrogen like compound. The method is used on a regular basis in many research laboratories including the laboratory at Environmental Medicine, University of Southern Denmark. The MCF-7 cell proliferation assay is considered to be the most reliable *in vitro* assay. It is of more humane physiologic relevance than other established *in vitro* assays as it is based on a mammal cell line which has not been manipulated genetically making the regulation of the estrogenic response more naturally (Andersen *et al.*, 1999).
In short, MCF-7 BUS cells were seeded in microtiter plates and after 24 h, dilutions (0.001-150 µM) of the azole fungicides were added for six days of incubation. The azole fungicides were tested with and without 10 pM 17β-estradiol (which induces half of the maximum proliferation response in the cells) to detect both antagonistic and agonistic effects. Cells were fixed and stained. The number of living cells was measured using an ELISA-reader. Each experiment included a standard curve of 17β-estradiol ranging from 1 pM to 10 nM and the maximum response was detected at 1 nM. The assay is very sensitive with a limit of detection of 0.5 pM 17β-estradiol and a quantification limit of 1 pM 17β-estradiol. Only results obtained with a standard curve slope within two times the standard deviation (estimated from a large number of independent experiments) was accepted. Furthermore, the mean control activity (based on minimum 3 wells) in each plate had to be within two times the standard deviation of controls to be included. This ensured a normal response from the cells and excluded increased background activity due to contamination. Each azole fungicide was tested in at least three independent experiments and in twenty-two different concentrations (0.001,

0.005, 0.01, 0.05, 0.1, 0.2, 0.39, 0.5, 0.78, 1, 1.56, 3.13, 5, 6.25, 10, 12.5, 30, 50, 75, 100, 125, 150 µM) in triplets. Results are presented as the Relative Proliferative Effect (RPE) in which data are normalized both to untreated control cells and the maximum response induced by 1 nM 17β-estradiol in each experiment:
$RPE = [(PE_{test\ sample}-1)/(PE_{max17\beta\text{-}estradiol}-1)] \times 100$, where PE correspond to the response normalized in relation to control. The concentration-response relationships were plotted and fitted to the sigmoidal function:
$y=y_0+a/[1+(x/x_0)^b]$

2.1.2 Aromatase testing – MCF-7 cells

The MCF-7 cells express the enzyme aromatase naturally. The enzyme converts testosterone to estrogen and hence induce proliferation of the cells (Sadekova *et al.*, 1994;Sonne-Hansen and Lykkesfeldt, 2005). By co-treating the cells with testosterone and fungicides an inhibition or stimulation of the enzyme activity can be registered (Almstrup *et al.*, 2002). Previously, the assay has been used (using 1 µM testosterone which induces about 65% of the maximum response of 17β-estradiol) for testing effects on aromatase activity by the fungicide fenarimol (Andersen *et al.*, 2006). The results obtained were in good agreement with results obtained using other aromatase assays. Prochloraz has been demonstrated to inhibit aromatase activity in different *in vitro* assays (Andersen *et al.*, 2002;Vinggaard *et al.*, 2000). By including prochloraz in this study it is possible to confirm the sensitivity of this assay compared to other assays. Since the same assay system is used for investigating estrogenicity and effects on aromatase it is possible to compare directly the concentrations inducing these effects.
Each azole fungicide was tested in three independent experiments at sixteen different concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 25, 30, 40, 50, 75, 100 µM) in triplets. Results are presented as RPE.

2.1.3 Cytotoxicity inMCF-7 cells

Cytotoxicity of the fungicides was evaluated by using the Promega Cytotox 96 Non Radioactive Cytotoxicity assay. Briefly, the MCF-7 cell proliferation assay was set up as usual for testing of estrogenicity or aromatase activity but with identical plates in pairs as the only difference. This was done to be sure that the assay passed as expected at the same time as the cytotoxicity could be measured. On the sixth day of exposure half of the plates were stopped as normal and the other half continued in the cytotoxicity assay.

The cytotoxicity was assessed indirectly by measuring the stable cytosolic enzyme lactate dehydrogenase (LDH) released from damaged cells before and after lysis. The LDH activity was measured by an enzymatic assay where tetrazolium salt converts into a red formazan product and where the subsequent measured absorbance then corresponds to the number of lysed cells (Promega, 2001). The more cytotoxic a compound is the more LDH will be released during the six incubation days of the assay and the less will be released after lysis. The maximum release corresponds to the total release of LDH before and after lysis. The cytotoxicity was assessed by calculating the ratio:

$$\%\ \ cytotoxicity\ \ = \frac{LDH_{spontaneous\_release}}{LDH_{maximum\_release}} * 100$$

Data will not be shown, but indicated in the relevant figures.

2.1.4 Androgen/anti-androgen testing – AR-assay

Effects on AR activity were tested in a reporter gene assay (Vinggaard *et al.*, 1999) with minor modifications. Chinese Hamster Ovary cells (CHO K1) were maintained in DMEM/F12 (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma-Aldrich, St.Louis, MO) and 10 % fetal bovine serum (FBS; BioWhitaker, Walkersville, MD). The cells were seeded in white 96-well microplates (PerkinElmer Life Sciences**,** Packard) at a density of 7,000 cells per well in DMEM/F12 containing 10 % charcoal-treated FBS (BioWhitaker) and incubated at 37°C in a humidified atmosphere of 5% CO_2/air. After 24 h cells were transfected for 5 h with a total of 75 ng DNA per well consisting of the expression vector pSVAR0 and the MMTV-LUC reporter plasmid (both provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam) in a ratio of 1:100 using 300 nl of the transfection reagent FuGene (Boehringer Mannheim, Germany). The ratio of DNA (µg) to Fugene (µl) was kept at 0.25. The fungicides were tested in twelve concentrations ranging from 0.025 to 50 µM, combined with 0.1 nM of the AR agonist R1881 (NEN, Boston, MA). The test solutions were prepared from 10 mM stock solutions in ethanol. After incubation for 20 h, the media was aspirated and the cells were lysed by adding 20 µl per well of a lysis buffer containing 25 mM trisphosphate, pH 7.8, 15 % glycerol, 1 % Triton X-100, 1 mM DTT and 8 mM $MgCl_2$, followed by shaking at room temperature for 10 min. Luciferase activity was measured directly using a Lumistar Galaxy luminometer by automatically injection of 40 µl substrate containing 1 mM luciferin (Amersham Int., Buckinghamshire, U.K.) and 1 mM ATP (Boehringer Mannheim, Germany) in lysis buffer and the chemiluminiscense generated from each well was measured over a 1 sec interval.

Cytotoxicity experiments were performed as described above, for the cell-procedures and transfections, except that the pSVAR0 expression vector was replaced by the constitutively active AR expression vector pSVAR13 (a gift from Brinkmann), which lacks the ligand-binding domain of the receptor. The ratio between pSVAR13 and MMTV-LUC was 2:100.

2.1.5 Ah receptor testing – CALUX assay

The rat hepatoma H4IIE cells were stably transfected with the PAH/HAH-inducible luciferase expression vector pGudLuc1.1. This vector contains the firefly luciferase gene under PAH/HAH-inducible control of four murine DREs (dioxin responsive elements) inducing luciferase in a time- and dose-dependent manner (Garrison *et al.*, 1996).

H4IIE cells were grown in α-MEM medium with 5% FBS at 37˚C in 95% air and 5% CO_2. Cells were seeded into sterile 96-well plates at 22.1 x 10^4 per ml and plates were incubated for 24 h prior to compound exposure, allowing cells to reach 90-100% confluence. Cells were exposed for 24 h to the azole fungicides (0, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 µM) in α-MEM supplemented with 1% FBS. Following exposure, cells were washed twice with PBS (pH 7.4) and lysed in 20 µl lysis buffer. An aliquot of 10 µl supernatant was pipetted into a 96-well microtitre plate and luciferase activity was determined using a Lumistar Galaxy luminometer.

2.1.6 Steroid synthesis testing – H295R cells

The H295R cell line, which is derived from human adrenocortical carcinoma cells, produces a wide range of steroid hormones in measurable quantities, including testosterone, progesterone and estradiol. This property makes the cell line suitable as a screening assay to detect effects on steroidogenesis. One alternative to the H295R cell line is the mouse Leydig tumour cell line MLTC-1. It produces progesterone, but only small amounts of testosterone and estradiol, which makes it less suitable as a screening assay to detect chemicals affecting steroid synthesis.
H295R cells (ATCC, CRL-2128) established from a pluripotent human adrenocortical carcinoma cell line were grown in 24-well culture plates (Costar, Corning, NY, USA) at 37°C humidified atmosphere of 5% CO_2/air. Each well contained 1 ml DMEM/F12 medium (GibcoBRL Life Tachnologies, Paisley, UK) supplemented with 2.0% Nu-serum (BD Sciences, Denmark), ITS+ premix (containing 6.25 µg/ml insulin, 6.25 g/ml transferin, 6.25 µg/ml selenuim, 1.25 µg/ml BSA and 5.35 µg/ml linoic acid) and 100 U/ml penicillin, 100 mg/ml streptomycin and 250ng/ml amphotericin B (Fungizone®). The cells were plated at a density of $2x10^5$ cells/well and allowed to settle for 24 h. Culture medium was removed and new medium containing fungicide dissolved in dimethyl sulfoxid (DMSO) was added to the cells in triplicates (prochloraz: 0, 0.01, 0.03, 0.1, 0.3, 1, and 3 µM, epoxiconazole, propiconazole, and tebuconazole: 0, 0.1, 0.3, 1, 3, 10 and 30 µM). Control wells contained the same amount of DMSO (0.1%) as exposed cells. After incubation for 48 h, the medium was removed and stored at -20°C until measured for testosterone, progesterone and estradiol levels. Samples of 800 µl were concentrated on IST Isolute® SPE columns (100mg, C18, 1ml) (Hengoed, UK), and the steroid hormones were measured using Delfia kits (PerkinElmer Life Sciences, Turku, Finland).

After exposure, the cells were incubated with resazurin solution to test for cytotoxicity. Medium from these wells (200µl) was transferred to black microplates (Costar, Corning, NY, USA) before fluorescence was measured. No cytotoxicity was observed for any of the concentrations used.

2.1.7 Thyroid testing – GH3 thyroid assay (T-Screen)

For the *in vitro* detection of agonistic and antagonistic properties of the azole fungicides towards the TR, the relatively new test method, called the T-screen, was used. The assay is based on the thyroid hormone dependent cell growth of a rat pituitary tumor cell line (GH3). This cell line expresses intracellular TR in very high amounts, and the assay can be used to study interference of compounds with thyroid hormone at the cellular level.

The GH3 cells were cultured at 37˚C humidified atmosphere of 5% CO_2/95% air in DMEM/F12 (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma-Aldrich, St.Louis, MO) and 10 % fetal calf serum (FCS)(Gibco, UK). Forty-eight hours prior to plating the cells into 96-well microplates (Costar, Fisher Scientific Biotech Line) for the experiment, standard culture medium was changed to test medium containing 10% (v/v) T_3- and T_4-depleted dextran-charcoal treated FCS (DC-FCS). After 48 h in DC-FCS, which was changed once after 24 h, the GH3 cells were released using a cellscraper and seeded in 96-well black, clear bottom microplates, 50 µl cell suspension and 50 µl test compound per well, at a density of 2500 cells/well. The azole fungicides were

dissolved in DMSO, and all test solutions were prepared from 20 mM stock solutions, except the T_3 stock solution, which was 10 mM. All four azole fungicides were tested in triplicates (epoxiconazole, prochloraz, propiconazole, and tebuconazole: 0, 0.01, 0.375, 1, 3, 10 and 30 µM) and were tested both in the absence and presence of 0.25 nM T_3 (the reported T_3-EC_{50} by (Gutleb *et al.*, 2005), to test for agonistic and antagonistic potency. Control wells contained cells and test medium with the same amount of DMSO (0.1%) as the exposed cells. The plates were incubated for 96 h, and cell growth was analyzed using AlamarBlue™ (BioSource, California, USA). 10% (v/v) AlamarBlue was added to each well, and the plates were incubated 4 h at 37˚C, protected from light. The experiment was terminated and the plates were analyzed by measuring fluorescence (ex. wavelength 560 nm/em. 590 nm) on a Wallac Victor 1420 multilabel counter (PerkinElmer life Sciences, Turku, Finland).

2.2 *In vivo* testing

The second step in the investigation of endocrine disrupting properties of azole fungicides was to include animal studies. First a short-term animal study was conducted to study whether the azole fungicides were anti-androgenic in young adult rats. The results obtained in this study were used to design the following study in which pregnant rats were exposed to the fungicides to investigate endocrine disrupting effects on fetuses and offspring. This later assay is presently considered the most sensitive, but also a very comprehensive animal study, for testing of endocrine disruptors.

2.2.1 Anti-androgenic testing – Hershberger test

Male Wistar rats were acquired from Taconic M & B, Eiby, Denmark. 6 intact male rats (42 days old at the dosing start) and 54 males, castrated at an age of 4 weeks, 14 days prior to study start, were used. All animals were delivered 1 week prior to study start and were upon arrival housed in Bayer Makrolon type 3118 cages (Type: 80-III-420-H-MAK, Techniplast), three per cage with Tapvai bedding. They were fed Syn 8.IT (a diet known to be free of phytoestrogens) and were provided with acidified tap water *ad libitum*. Animal rooms were maintained on a 12-hour light/dark cycle, a temperature of 22 ± 1 °C and a relative humidity of 55 ± 5 %. Rats were weighed and divided by randomization into treatment groups so that there were no statistically significant differences among group body weight means. During testing rats were weighed daily and visually inspected for health effects twice a day.

One group of intact animals and 9 groups of castrated male Wistar rats, were included in the study (n=6 per group). The intact rats and the one group of castrated rats served as negative controls and were given peanut oil only. The rats in the remaining eight groups were all dosed with testosterone propionate (0.5 mg/kg/day *sc*). One group was only administered testosterone propionate and served as control group to which all other groups were compared. The positive control group received flutamide (10 mg/kg bw/day orally). The last 6 groups received propiconazole or tebuconazole orally at doses of 50, 100, or 150 mg/kg bw/day.

All compounds were dissolved in peanut oil. Sterile oil (the Royal Veterinary Agriculture Pharmacy, Copenhagen, Denmark) was used for the testosterone

propionate solution. All compounds were administered in a dosing volume of 2 ml/kg body weight and the dosing period was 7 days for all animals. The testosterone dose was always given a few minutes after the test compound and the last dosing was performed in the morning at the day of killing the animals. Body weights were recorded and animals were euthanized using CO_2/O_2 followed by exsanguinations. All the animals from each group underwent a thorough autopsy. The testis (for intact animals), both lobes of the ventral prostate, combined seminal vesicles and coagulating glands including fluids, levator ani/bulbocavernosus muscle (LABC), paired bulbourethral glands, pituitary, liver and paired kidneys were dissected and weighed. Organ weights were calculated relative to body weights. The ventral prostates were put in 0.5 ml RNAlater (Ambion) and stored at -20°C until gene expression analysis. Blood was collected by heart puncture in plain glass tubes and serum was prepared and stored at -80°C until measurement of hormones.

2.2.1.1 Hormone levels

Rat luteinizing hormone (LH), rat follicle stimulating hormone (FSH), and T_4 levels were analyzed in serum using the technique of time-resolved fluorescence (Delfia, Wallac). LH and FSH were analyzed at Turku University, Finland. Rat FSH immunoreactivity was determined by a two-site immunofluorometric assay (IFMA) (van Casteren *et al.*, 2000). The standard used was a NIDDK standard FSH-RP-2 obtained from the National Hormone and Pituitary Program, NIH, Rockville, MD.
Rat LH was measured using the time-resolved fluorimetric assay (IFMA, Delfia, Wallac OY, Turku, Finland) as described (Haavisto *et al.*, 1993). The standard rLH RP-3 was kindly provided by NIDDK, NIH (Baltimore, MD).

2.2.1.2 Gene expression levels determined by real-time RT-PCR

The organs were homogenized and total RNA was isolated using RNeasy-mini kit and RNase-Free DNase set (Qiagen). cDNA was synthesized from 0.5 µg total RNA using the Omniscript Reverse Transcription kit (Qiagen) with T16 oligoes and a 18S rRNA primer. Samples were quantified on the 7900HT Fast Real-Time PCR System (Applied Biosystems) by standard TaqMan technology. Expression levels of the following genes were quantified, in the ventral prostate: The androgen-responsive genes prostate binding protein C3 (PBP C3), ornithine decarboxylase (ODC), testosterone-repressed prostate message 2 (TRPM-2), complement component 3 (Compl. C3), the androgen receptor (AR) and insulin-like growth factor 1 (IGF-1). These genes were chosen because they have previously been shown to be androgen-regulated. PBP C3 is a tissue-specific protein and the principal secretory protein in rat prostatic fluid (see references in (Nellemann *et al.*, 2001)). ODC is necessary for cell growth and differentiation as an important enzyme in the synthesis of polyamines. ODC catalyzes the conversion of ornithine to putrescine, which is the first and rate-limiting step in polyamine biosynthesis. TRPM-2 also called clusterin, is not normally expressed in the rat ventral prostate, but is induced by castration (Leger *et al.*, 1987). TRPM-2 is expressed at high levels in dying cells, and data suggest that TRPM-2 acts as a survival factor from apoptosis (Ho *et al.*, 1998;Ogawa *et al.*, 2005;Viard *et al.*, 1999). Compl. C3 is involved in the innate immune response and together with PBP C3, ODC and TRPM-2, this gene has been selected, because the expression of these four genes in ventral prostate, previously have been found to be valuable for investigating anti-androgenic effects in the Hersberger assay (Nellemann *et al.*, 2001;Nellemann *et al.*, 2005). For each sample, 2 µl cDNA (1.75 ng/µl) was amplified under universal thermal cycling parameters (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix

(Applied Biosystems) in a total reaction volume of 10 µl. Three separate amplifications were performed for each gene and when intra-assay variation was above 10% additional amplifications were performed. All genes were quantified from standard curves, and expression levels of each target gene were normalized to the expression level of the housekeeping gene 18S ribosomal RNA (18S rRNA). Table 1 illustrates the sequences of the primers used.

Table 1 - Sequences of the primers used for quantitative real-time PCR.

18S rRNA
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'
PBP C3
5'-FAM-TCA TCT 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'
ODC
5'-FAM-ACT CAC TGC TGT AAC ACA CAG CCT GTG CA-TAMRA-3'
Forward, 5'-AAT GTG TGC AAG TAT CCC TTA CAG AA-3'
Reverse, 5'-CAC AGC TTT GTA TCA TCC ACA TCT C-3'
TRPM-2
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'
Compl.C3
5'-FAM-CGT AGT CCA CTC CAG GCT CAC AAG-TAMRA-3'
Forward, 5'-CAG CCT GAA TGA ACG ACT AGA CA-3'
Reverse, 5'-AAA ATC ATC CGA CAG CTC TAT CG-3'
IGF-1
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
5'-FAM-TCG CGA TTC TGG TAT GCT GCT GC-TAMRA-3'
Forward, 5'- GAC ACT TGA GAT CCC GTC CT-3'
Reverse, 5'- GAG CGA GCG GAA AGT TGT AG-3'

2.2.2 Developmental effects on offspring after perinatal exposure

To test for developmental effects, pregnant dams were dosed with epoxiconazole or tebuconazole and the fetuses and offspring were examined for effects on sexual differentiation. Important biological endpoints were: gestational length, survival, anogenital distance (AGD) in fetuses and offspring, retention of nipples in male pups, decreased weight of accessory sex tissues, semen quality, morphological effects on the external genitalia (hypospadia and cryptorchidism), hormone levels, and gene expression. Figure 2 is a schematic overview of the study design.

2.2.2.1 Animals and exposure

In short, 112 pregnant Wistar rats (HanTac: WH, Taconic M& B, Ejby, Denmark) were received on gestational day (GD) 3. The day after arrival, the animals were weighed and assigned to treatment groups (1-5) by stratified randomization to assure equivalent body weight means across groups prior to dosing. The rats were gavaged peanut oil (n=24), with 15 or 50 mg/kg bw/day epoxiconazole (n=24/24), or 50 or 100 mg/kg bw/day tebuconazole (n=20/20) respectively, from GD 7 to postnatal day (PND) 16. Animals were divided into two sets, such that 56 animals representing all 5 groups were dosed one week prior to the next 56 animals. In the 5 groups 19, 19, 18, 19 and 21 rats, respectively, were pregnant.

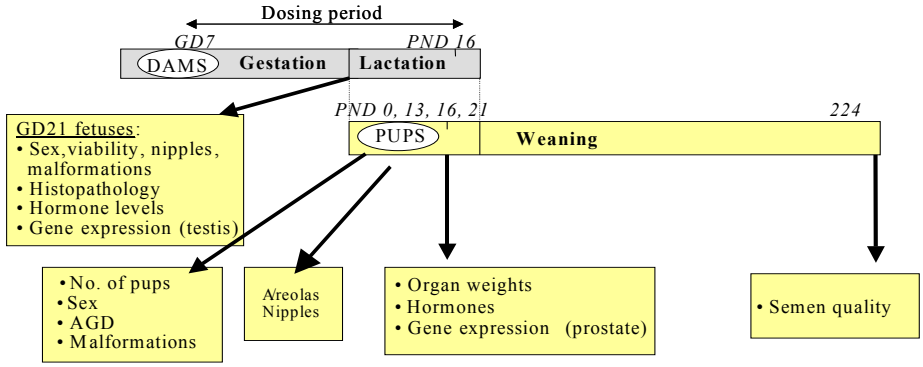


Figure 2 - Schematic overview of the design of the developmental toxicity study. Pregnant dams were dosed GD7 to PND 16. At GD 21 some of the dams were chosen for caesarian section and the fetuses were investigated. Some of the dams were allowed to give birth (PND 0). At PND 16 some of the offspring were killed and tissue was taken for hormone- and gene expression analysis as well as pathology. Some of the offspring continued in the study for behavior tests (not included in this report) and semen quality tests (PND 224).
GD: gestational day; PND: postnatal day; AGD: anogenital distance.

2.2.2.2 Health status of dams and delivery

The rats were observed twice daily for signs of toxicity. Body weight was registered daily from GD 7 to PND 16. The maternal weight gain from GD 7 to GD 21, GD 7 to PND 1, and PND 1 to PND 13 was calculated from the data. The first measure is based on the weights of the dams including the weight of the fetuses and may therefore, if affected, reflect an effect on the maternal animal and/or the fetuses. In contrast, the maternal weight gain from GD 7 to PND 1 as well as the maternal body weight on PND 1 provides measures of the dam body weight only. From GD 21 the rats were inspected twice a day to register time of birth. After delivery body weights of dams and pups were recorded. Number of pups, sex ratio and anomalies were registered. The day of delivery is PND 0.

2.2.2.3 Caesarian sections GD 21

At GD 21 the following numbers of dams were originally selected for Caesaeian section: 6 controls; 7 dosed with 50 mg/kg bw/day and 8 dosed with 100 mg/kg bw/day tebuconazole; 9 dosed with 15 mg/kg and 14 dosed with 50 mg/kg epoxiconazole. However, because of problems with parturition in the highest dose groups, caesarian section was additionally performed on 2 dams in the group receiving 100 mg tebuconazole/kg bw/day and on 4 dams in the group receiving 50 mg epoxiconazole/kg bw/day group. The dams were

weighed and decapitated after CO_2/O_2 anesthesia. Uterus was taken out, and the number of live fetuses, re-absorptions, implantations and any anomalies was registered. The remaining dams (13, 12, 8, 10 and 3 dams from the control, the 50 mg/kg and the 100 mg/kg tebuconazole, the 15 mg/kg and the 50 mg/kg epoxiconazole group, respectively) were allowed to give birth.

2.2.2.4 Section of pups PND 16

At PND 16 all mothers and all pups except 72 pups, were killed and tissue was taken for hormone-, gene expression-, and pathology analysis. The 72 remaining animals continued in the study for behavioral tests (not included in this report) and semen quality analysis (34 male pups).

2.2.2.5 Histology and Immunohistochemistry GD 21

In fetuses at GD 21 one testis from 1 to 3 males per litter were placed in Bouin's fixative, embedded in paraffin and one section per male was used for histopathology (haematoxylin and eosin stain) or immunohistochemistry. Immunohistochemistry was performed on one section per testis. Following microwave pretreatment for 2x5 min in either citrate or TEG buffer, sections were blocked for endogenous peroxidase activity in 3% H_2O_2 in phosphate buffered saline (PBS), and blocked in 1% bovine serum albumin in PBS. Sections were incubated over night at 4˚C with the following rabbit polyclonal antibodies: Steroidogenic acute regulatory protein (StAR) antibody (PA1-560, Affinity Bioreagents, Golden, CO, 1:1000), cytochrome P450-side-chain cleavage (P450scc) antibody (AB 1244, Chemicon, Temecula, CA, 1:50,000), peripheral benzodiarepine receptor (PBR) antibody (SantaCruz, CA, 1:200), or 17β-hydroxysteroid dehydrogenase (17β-HSD) type 10 (Kem-En-Tec). Sections were then incubated for 30 min with secondary antibody (Anti-rabbit EnVision+, DAKO, Glostrup, Denmark), stained in diaminobenzidine (DAB+ Substrate Chromogen System, DAKO, Glostrup, Denmark) or 3-amino-9-ethylcarbazole (AEC, Labvision, CA), and counterstained in Meyer's hematoxylin. Negative controls were fetal control testis, incubated with blocking serum instead of primary antibody.

2.2.2.6 Hormone analysis GD 21 and PND 16

Testosterone, progesterone, 17α–hydroxy-progesterone, estradiol and thyroid hormones were analyzed in rat plasma or testes at GD21 and/or PND 16. Steroid hormones were extracted from the serum on IST Isolute C18 SPE columns (200mg/3ml). The serum samples were diluted with purified water 1:1 and applied to columns, preconditioned and rinsed with methanol (MeOH) and water, respectively. Interfering substances were eluted with 2x2 ml MeOH:water (20:80 v/v) and steroids were eluted with 2x2.4 ml MeOH. The solvent in these fractions was evaporated and samples were resuspended in 100 µl Diluent 1 (PerkinElmer, Turku, Finland) and the steroids together with the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) were analyzed using a Delfia time-resolved fluorescence kit (PerkinElmer, Turku, Finland) and measured by use of a Wallac Victor 1420 multilabel counter (PerkinElmer life Sciences, Turku, Finland).

Steroid hormones were analyzed in testis and estradiol in ovaries after extraction with diethyl ether. Decapsulated testes or ovaries were placed in vials containing 100 or 500 µl water and 0.5 or 2.5 ml diethyl ether, respectively. The tissue was homogenized and the vials were placed in a tub consisting of dry ice and acetone until the water-fraction was frozen. The ether-fraction was transferred to a clean vial, the procedure was repeated, and the two extracts were pooled and evaporated. Before analyzing, the samples

were re-suspended in 100 µl Diluent 1 and incubated over night at 4˚C. The day of analysis the samples were vortexed and incubated for 10 min at 45˚C, before the hormones were measured by use of the Delfia kit as mentioned above.

Ex vivo testosterone and progesterone production at GD 21 was determined by decapsulating and incubating the testis in a shaking water bath at 34˚C for 3 h in DMEM/F12 medium containing 0.1 % BSA. Vials were centrifuged at 4000 x g for 10 min and the supernatants were stored at -80˚C until hormone levels were analyzed as mentioned above. Testosterone levels were measured in undiluted supernatant, but for the measurement of the progesterone levels the supernatants were concentrated 5 times by the use of IST Isolute C18 SPE columns and resuspended in 100 µl Diluent 1.

2.2.2.7 Gene expression levels GD 21 and PND 16

The following genes were studied in testes at GD 21: scavenger receptor class B, member 1 (ScarB1), StAR, P450scc and Cyp17a1 (P450c17). In the testis ScarB1 transports serum lipoproteins (HDL) into the testis Leydig cell, where they are used in the synthesis of cholesterol. StAR transports the cholesterol from the outer to the inner mitochondrial membrane where the first enzyme in steroid synthesis, P450scc, is located, which converts cholesterol to pregnenolone. P450c17 is involved in several of the steps that convert pregnenolone to testosterone, and functions both as a 17α–hydroxylase and a 17,20-lyase (Felig *et al.*, 1995) (Figure 3).

At PND 16 the following genes were studied in ventral prostate: AR, ODC, Compl. C3, IGF-1, PBP C3, and TRPM-2. In the epididymides the expression level of the placenta and embryos oncofetal gene (PEM) was quantified. PEM is a home-domain-containing protein, proposed to play a role in the regulation of androgen-dependent events in the epididymis.

Each sample was processed as previously described in section 2.2.1.2 and all genes were quantified from standard curves, and expression levels of each target gene were normalized to the expression level of the housekeeping gene 18S rRNA. See Table 2 for primer sequences used for the quantitative real-time PCR in testis and epididymis.

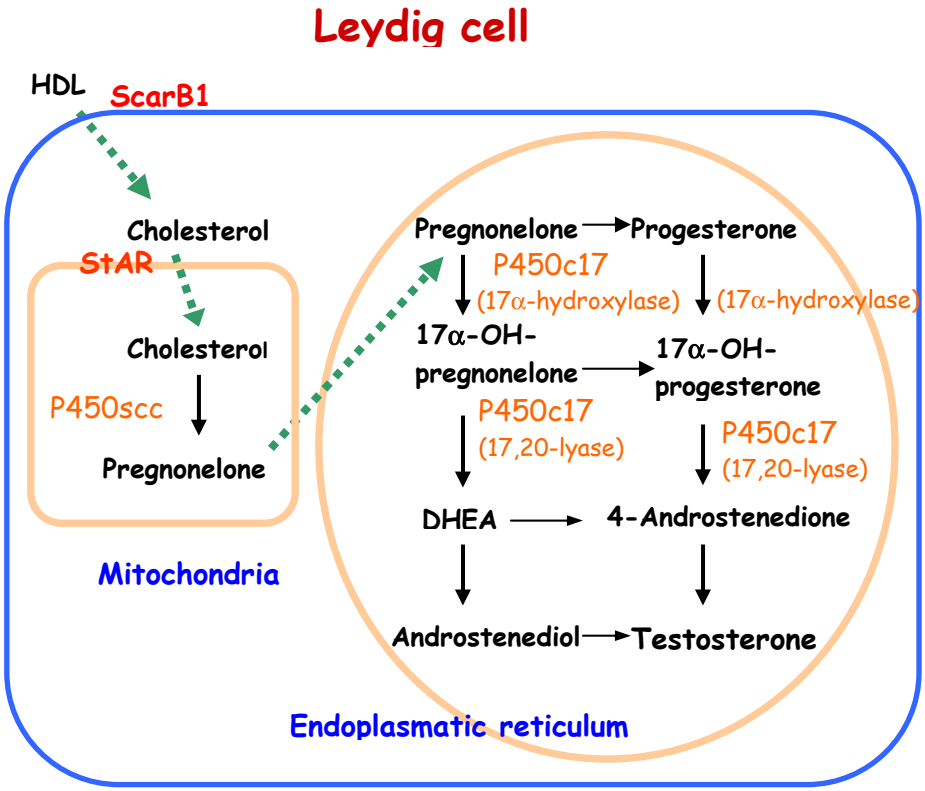


Figure 3 - Illustration of the pathway of testicular testosterone synthesis. The uptake of cholesterol and its conversion to testosterone involves numerous receptors and enzymes, including **ScarB1**, which is responsible for HDL uptake into Leydig cells, **StAR** that transport the cholesterol from the outer to the inner mitochondrial membrane and the steroid-converting enzymes **P450scc** and **P450c17**, which are involved in the conversion of cholesterol to testosterone.

2.2.2.8 Anogenital distance and nipple retention

AGD was measured in the offspring at birth (PND 1) using a stereomicroscope. On PND 13, the male pups were examined for the presence of areolas/nipples, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. To correlate for the size of the pups their body weights were measured at both time points (PND 1 and PND 13). AGD is expressed per cube root of body weight since this ratio has been demonstrated to be the most appropriate metric for normalizing of the data (Gallavan, Jr. *et al.*, 1999).

2.2.2.9 Autopsy of offspring on PND 16

The external genitalia were inspected blinded to the observer at PND 16 in all males from all litters. The changes were scored on a scale from 1 to 4 in order to investigate whether male external genitals were feminized. The following criteria were used: Score 1: No effect: normal genital tubercle, the urethral opening is found at the tip of the genital tubercle and the preputial skin is intact. In the perineal area, thick fur extends caudally from the base of the genital tubercle half the distance to the anus. A furless area circumscribes the anus. Score 2: Mild dysgenesis of the external genitalia: A small cavity on the caudal surface of genital tubercle and a minor cleft in the preputial opening is observed, estimated 0.5-1.4 on an arbitrary scale. The furless area around anus expands towards the base of the genital tubercle, but thick fur is still present at the base of the genital tubercle. Score 3: Moderate dysgenesis of the external genitalia: the preputial cleft is larger, estimated 1.5-2.4 on an arbitrary scale. The urethral opening is situated half way down the inferior

side of the genital tubercle (hypospadia). Lack of fur or thin fur in the perineal area ranging from the base of the genital tubercle and caudally to the furless area circumscribing the anus. Score 4: Severe dysgenesis of the external genitalia: The preputial cleft is large, estimated 2.5-3.5 on an arbitrary scale. The urethral opening is situated further than half way down the inferior side of the genital tubercle to the base of the genital tubercle. At the base of the genital tubercle a groove extending laterally is observed, and they are totally furless in the whole perineal area.

Table 2 - Sequences of the primer used for quantitative real-time PCR in testis and epididymis.

ScarB1
5'-FAM- AAA GCA TTT CTC CTG GCT GCG CAG-TAMRA-3'
Forward, 5'-TCT GGT GCC CAT CAT TTA CCA-3'
Reverse, 5'- AGC CCT TTT TAC TAC CAC TCC AAA-3'
StAR
5'-FAM- CTG ACT CCT CTA ACT CCT GTC TGC CTA CAT GGT-TAMRA-3'
Forward, 5'- CCC TTG TTT GAA AAG GTC AAG TG-3'
Reverse, 5'- TGA AAC GGG AAT GCT GTA GCT-3'
P450scc
5'-FAM- CCT TTA TGA AAT GGC ACA CAA CTT GAA GGT ACA-TAMRA-3'
Forward, 5'- ACG ACC TCC ATG ACT CTG CAA T-3'
Reverse, 5'- CTT CAG CCC GCA GCA TCT-3'
P450c17
5'-FAM- CGT CAA CCA TGG GAA TAT GTC CAC CAG A-TAMRA-3'
Forward, 5'-GCC ACG GGC GAC AGA A-3'
Reverse, 5'- CCA AGC CTT TGT TGG GAA-3'
PEM
5'-FAM- CCATCTATCAAGCTCCTCCCGCCACT-TAMRA-3'
Forward, 5'- CAT TTT GCT AAG CAG TGG TTC CT-3'
Reverse, 5'- CCT GCA CTC TGG ACA CAC TGA-3'

For the primer sequences used in the quantification of: 18S rRNA, PBP C3, ODC, TRPM-2, Compl.C3, IGF-1 and AR see Table 1.

2.2.2.10 Organ weight and histopathology PND 16

Body weights of all male pups were recorded. In one to two male per litter the following organs were excised and weighed: liver, kidneys, adrenals, testes, epididymides, seminal vesicles, ventral prostate, bulbourethal glands, and LABC. In the analysis of body and testis weight, generally one to four males per litter were used.

From one or two males per litter, the right or left testes were alternately fixed in Bouin's fixative, paraffin embedded, and stained with hematoxylin and eosin. In one male per litter, the following organs were fixed in formalin: ventral prostate, seminal vesicles, and epididymides. All fixed organs were embedded in paraffin and examined by light microscopy after staining with hematoxylin and eosin and used for the histopathological evaluation.

In one to two females per litter, body weights were recorded. The thyroid, ovaries and uterus were excised and weighed from one female per litter.

2.2.2.11 Semen quality analysis PND 224

Due to toxicity in the highest dose groups, none of the high dose animals were available for the semen quality analysis. Semen quality was therefore only analyzed in the controls and the low dose groups of tebuconazole and epoxiconazole, using 11-12 male offspring per group at the age of approximately 7 months. The animals were anaesthetised by CO_2/O_2 and decapitated. The epididymis were removed and the cauda of the right epididymis was used for sperm motility analysis.

2.2.2.12 Sperm motility

The epididymis was trimmed of fat and cut at the corpus-cauda region. The cauda was placed in a petri dish containing 3 mL warm (37ºC) Medium 199, supplemented with 0.5% bovine serum albumin (crystallized and lyophilized; Sigma Chemicals Company, USA). Spermatozoa were obtained from the distal cauda where the tubular diameter was widest. Under the dissecting microscope, the cauda was held by forceps and several stabs were made into the tubules. The petri dish was placed in an incubator for 5 min. The cauda was removed and the sperm sample was diluted 10 times (in medium 199, supplemented with 0.5% bovine serum) and replaced in the incubator for 10 min, to allow dispersion of the spermatozoa. The sperm sample was loaded into a 100-µm flat cannula (HTR 1099, DIPL.ING. HOUM, Norway) and analyzed by computer assisted sperm analysis (CASA; HTM-IVOS version 10.6, Hamilton Thorne Research, Beverly, MA, USA). Several fields (minimum 20) were recorded at 60 Hz under 4× dark field illumination, and the images were video recorded for later analysis.

The standard set-up was used during analysis and tracking errors were deleted through the edit and playback features. Twelve fields (minimum 200 sperm cells) were analyzed for each sperm sample. The parameters evaluated in this study were percent motile and percent progressive spermatozoa, curvilinear velocity (VCL), and amplitude of lateral head displacement (ALH), which describes the vigour of the spermatozoa, and progression parameters such as average path velocity (VAP), straight-line velocity (VSL), and straightness (STR).

2.3 Statistical analyses

2.3.1 *In vitro* and *in vivo* Hershberger test

Data did not deviate from normality or homogeneity of variance. Data were analyzed by one-way ANOVA followed by pair-wise comparisons between test and control groups using Dunnetts test. Significance was judged at $p<0.05$.

2.3.2 Developmental Study

The litter was generally considered the statistical unit and the alpha level was 0.05. The results were analyzed by analyses of variance (ANOVA), and in order to adjust for litter effects, litter was included in the analysis of variance as a nested factor.

For statistical evaluation of testosterone and progesterone levels in testes (2 - 4 males per litter) and *ex vivo* testosterone and progesterone production GD 21 (3 -11 males per litter), all males were included in the analysis. Data from one

male and one female per litter at PND 16 (12-16 litters per group) and one or two males per litter at PND 224 (11-16 litters per group leading to a total of 16 animals per group) were used to analyze terminal body weight and organ weights. In order to adjust for litter effects, litter was included in the analysis of variance as an independent, random, and nested factor (proc mixed, SAS version 8, SAS Institute Inc, Cary, NC, USA). Organ weights were analyzed using treatment as one main factor and age as another main factor and body weight was used as a covariate. Non-processed and ln-transformed data were examined for normal distribution and homogeneity of variance. If an interaction between age groups and dose group was observed, the age groups were analyzed separately. When an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. In cases where normal distribution and homogeneity of variance was not obtained, data were additionally tested with the non-parametric Wilcoxon Scores followed by a Kruskall-Wallis test.

Plasma hormone data were anayzed by a one-way ANOVA and, if significant, followed by Dunnett's test. Significance was judged at $p < 0.05$. Sperm data were examined for normal distribution and homogeneity of variance. Single animal data were analyzed in a one-way analysis of variance (proc glm) followed by Dunnett`s t-test (version 8, SAS Institute Inc, Cary, NC, USA).

3 Results - Endocrine disrupting effects of azole fungicides

3.1 *In vitro* effects

To investigate the endocrine disrupting mechanisms involved, the four azole fungicides: epoxiconazole, prochloraz, propiconazole, and tebuconazole were tested in a range of *in vitro* test systems to evaluate anti/estrogenicity, anti/androgenicity, dioxin like effects, disruption of the thyroid function, and effects on aromatase activity and other enzymes involved in the steroid synthesis.

3.1.1 Estrogenic/anti-estrogenic effects of azole fungicides

Figure 4 illustrates that all four fungicides inhibited MCF-7 cell proliferation induced by 10 pM 17β-estradiol and hence exhibited weak anti-estrogenic responses. The response was statistically significant from 1.6 µM (Table 3). Concentrations causing 25%, 50%, and 75% inhibition of the 17β-estradiol induced (10 pM) MCF-7 cell proliferation response (IC_{25}, IC_{50}, IC_{75}) were calculated (Table 3). IC_{50} values were found to be 49, 28, 52, and 45 µM for epoxiconazole, prochloraz, propiconazole, and tebuconazole, respectively. Prochloraz was the most potent of the four fungicides.

Table 3 - Concentrations (µM) of the fungicides causing 25, 50, or 75 % inhibition of the 17β-estradiol (10 pM) induced MCF-7 cell proliferation response.

	Epoxiconazole	Prochloraz	Propiconazole	Tebuconazole
LOEC	25	12.5	25	1.6
IC_{25}	36	21	40	32
IC_{50}	49	28	52	45
IC_{75}	66	38	64	63

LOEC: Lowest observed effect concentration. LOEC is chosen as the lowest concentration causing a continuously statistically significant response.

Since a decreased proliferation can be due to general cell toxicity of the fungicides in the MCF-7 cells, a LDH-test for cytotoxicity was performed. Cytotoxicity was only detected at the highest azole concentrations (indicated by a c in the figures) and cannot explain the decreased cell proliferation of the azole fungicides at lower concentrations.

In addition, epoxiconazole and propiconazole increased the cell proliferation indicating a weak estrogenic activity (Figure 5). This activity was statistically significant at 6.25 and 25 µM, respectively.

Experiments with the well-known anti-estrogenic compound ICI 182.780 (1 nM) revealed that the cell proliferation induced by these fungicides was counteracted by ICI 182.780, thus, indicating that the proliferation is induced directly via the ER receptor (data not shown).

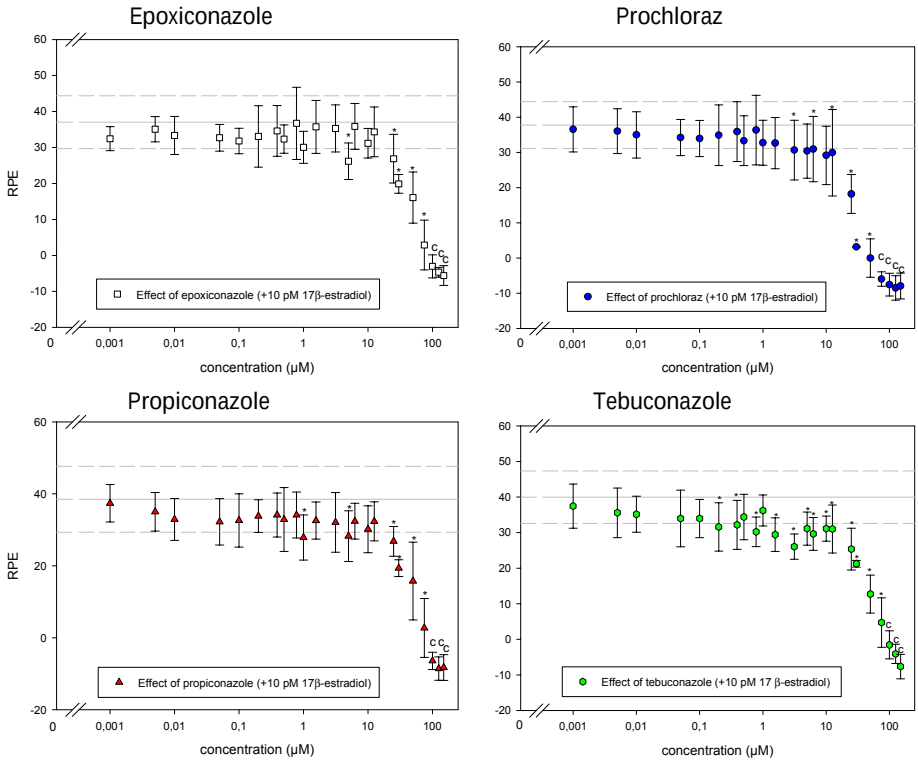


Figure 4 - Anti-estrogenic effects of the four fungicides: epoxiconazole, prochloraz, propiconazole, and tebuconazole tested in the MCF-7 cell proliferation assay. All four fungicides inhibited the proliferation of MCF-7 cells induced by 10 pM 17β-estradiol. Data represent mean±SD for three to seven independent experiments. The horizontal gray line represents the control level and the dashed gray lines represent the SD of the control. * Statistically significantly different from control (10 pM 17β-estradiol) ($p<0.05$). c = tested cytotoxic by use of a LDH-test. RPE = Relative Proliferative Effect.

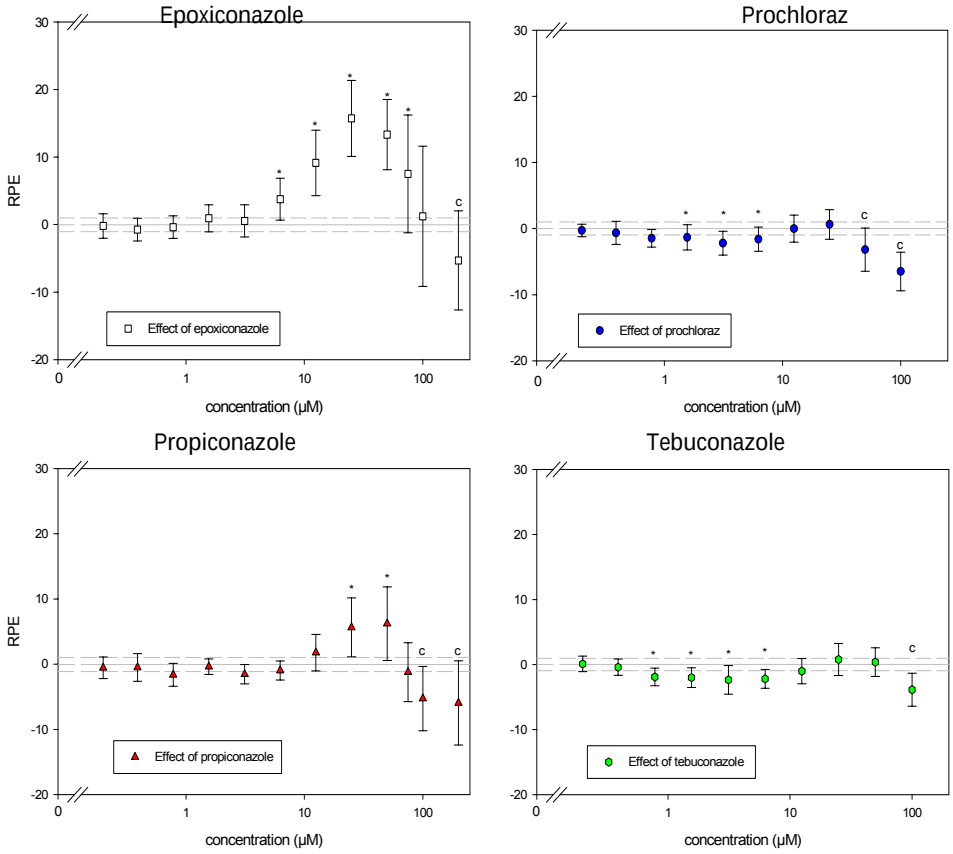


Figure 5 - Estrogenic effects of the four fungicides: epoxiconazole, prochloraz, propiconazole, and tebuconazole tested in the MCF-7 cell proliferation assay. Epoxiconazole and propiconazole induced proliferation of the MCF-7 cells. Data represent mean±SD for three to seven independent experiments. The horizontal gray line represents the control level and the dashed gray lines represent the SD of the control. ∗ Statistically significantly different from control ($p<0.05$). c = tested cytotoxic by a LDH-test. RPE = Relative Proliferative Effect.

3.1.2 Aromatase effects of azole fungicides

Figure 6 illustrates that all four fungicides inhibit the 1µM testosterone-induced MCF-7 cell proliferation. The effect is statistically significant from 1 µM. The response obtained in this assay will be the combined effect of aromatase inhibition and anti-estrogenicity as aromatase converts testosterone to estrogen, which then induces proliferation of the cells.

Concentrations causing 25%, 50%, and 75% inhibition of the 1 µM testosterone induced MCF-7 cell proliferation response (IC_{25}, IC_{50}, IC_{75}) were calculated (Table 4). IC_{50} values were found to be 17, 9, 29, and 19 µM for

epoxiconazole, prochloraz, propiconazole, and tebuconazole, respectively. Prochloraz was the most potent among the four (Table 4).

The concentrations of the azole fungicides needed to reduce the testosterone-induced response were lower than the concentrations needed to reduce the 17β-estradiol-induced response. This indicates that these compounds possess both aromatase inhibiting and anti-estrogenic properties but that aromatase inhibition dominates at low concentrations.

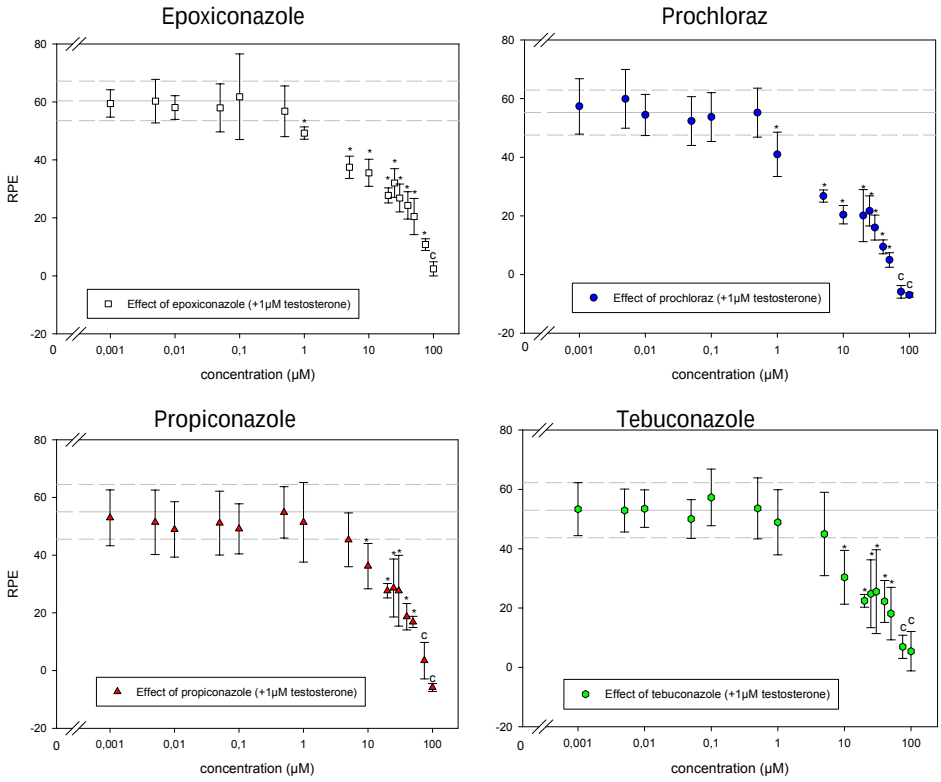


Figure 6 - Effects on the enzyme aromatase of the four fungicides: epoxiconazole, prochloraz, propiconazole, and tebuconazole tested in the MCF-7 cell proliferation assay. All four fungicides inhibited the 1 µM testosterone (converted by aromatase to estrogen) induced MCF-7 cell proliferation. Data represent mean±SD for three independent experiments. The horizontal gray line represents the control level and the dashed gray lines represent the sd of the control. ∗Statistically significantly different from control (1 µM testosterone) ($p<0.05$). c = tested cytotoxic by a LDH-test. RPE = Relative Proliferative Effect.

Table 4 - Concentrations (µM) of the fungicides causing 25, 50, or 75 % inhibition of the testosterone-induced (1 µM) MCF-7 cell proliferation response.

	Epoxiconazole	Prochloraz	Propiconazole	Tebuconazole
LOEC	1	1	10	10
IC_{25}	4	3	16	6
IC_{50}	17	9	29	19
IC_{75}	73	25	53	60

LOEC: Lowest observed effect concentration. LOEC is chosen as the lowest concentration causing a continuously statistically significant response.

3.1.3 Anti-androgenic effects of azole fungicides

All four azole fungicides proved to be AR antagonists. No cytotoxicity was observed except for prochloraz and tebuconazole that were cytotoxic at 50 µM (data not shown). As shown in Figure 7 all the azole fungicides had a potency of comparable magnitude. The LOECs were 0.8, 3.1, 25 and 3.1 µM for epoxiconazole, prochloraz, propiconazole, and tebuconazole respectively.

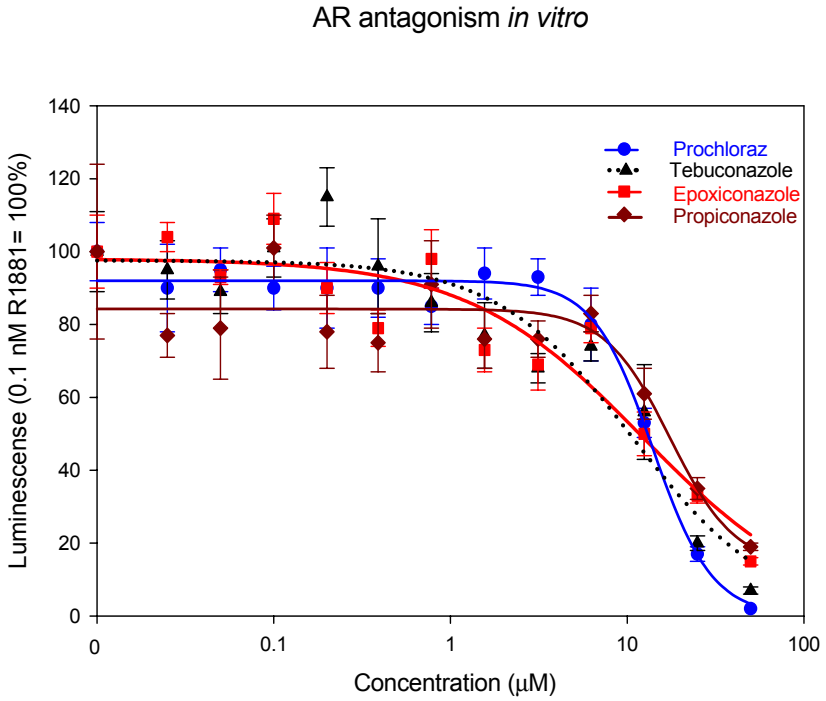


Figure 7 - Anti-androgenic effects determined in the AR reporter gene assay. The fungicides are tested in combination with the AR agonist R1881 in AR transfected CHO cells. Data represent mean±SD for three independent experiments. * Significance level $p < 0.05$ compared to the control (0.1 nM R1881).

3.1.4 Dioxin-like effects of azole fungicides – Ah-receptor testing

Like prochloraz (Long *et al.*, 2003) epoxiconazole, propiconazole, and tebuconazole were able to activate the Ah receptor (Figure 8), but far from the order of magnitude displayed by prochloraz. The LOECs were 6.3, 0.05, 12.5 and 6.3 µM for epoxiconazole, prochloraz, propiconazole, and tebuconazole, respectively (Table 5).

Table 5 - Concentrations (µM) of the fungicides needed to induce a significant response in the AhR CALUX reporter gene assay, and the effect of the fungicides in relation to the maximum response induced by the AhR agonist TCDD.

	Epoxiconazole	Prochloraz	Propiconazole	Tebuconazole
LOEC	6.3	0.05	12.5	6.3
MOEC	50	10	50	50
% Effect of max. TCDD effect	9	38	10	8

LOEC: Lowest observed effect concentration. LOEC is chosen as the lowest concentration causing a continuously statistically significant response. MOEC: Maximum observed effect concentration: the lowest concentration causing maximum response.

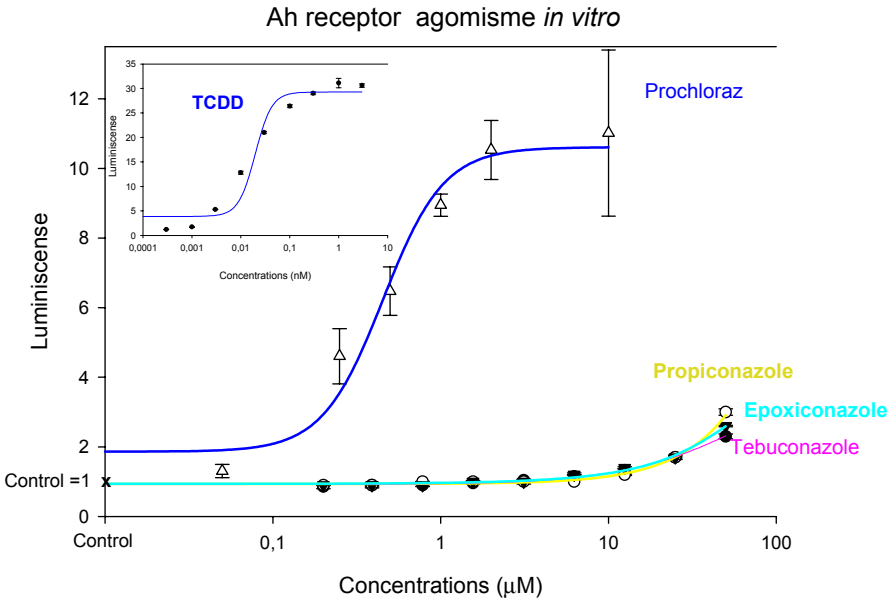
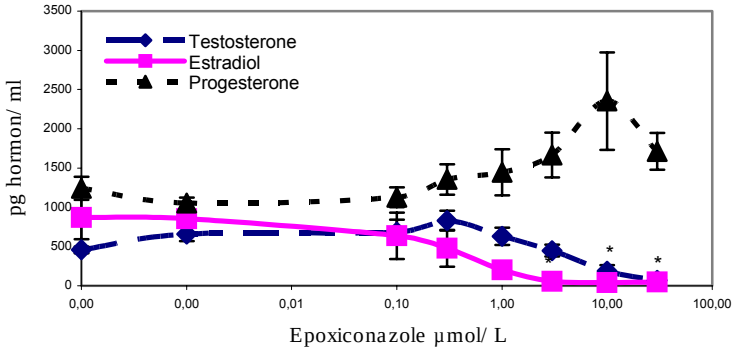


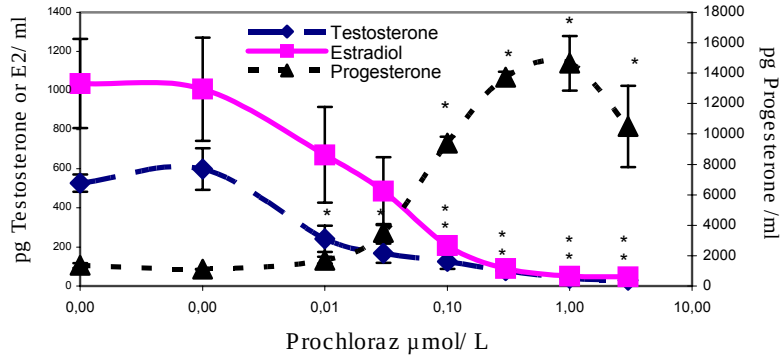
Figure 8 - Agonistic effect measured in the AhR CALUX reporter gene assay. Concentration response curves for the four fungicides epoxiconazole, prochloraz, propiconazole, and tebuconazole and for the AhR agonist TCDD. For concentrations above 10 µM a decrease of luciferase activity was seen for prochloraz, which is due to a cytotoxic effect. * The prochloraz data is from a previous conducted study in 2002.

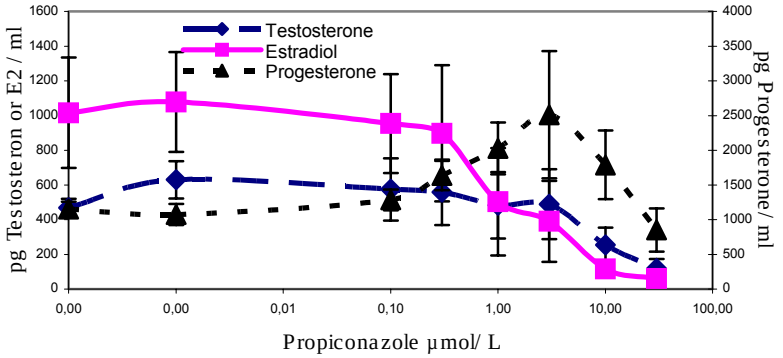
3.1.5 Steroid synthesis effects of azole fungicides

In order to look for effects on steroidogenesis *in vitro*, we tested epoxiconazole, prochloraz, propiconazole and tebuconazole in the H295R steroid synthesis assay, and compared it to the results of the previously conducted study with prochloraz (Laier *et al.*, 2006).

Epoxiconazole, propiconazole, and tebuconazole, were like prochloraz able to inhibit the production of testosterone and estradiol *in vitro* in H295R cells, though the inhibiting effect on testosterone production was only statistically significant for the two highest concentrations of tebuconazole. The estradiol production was statistically significantly inhibited at the two highest concentrations of tebuconazole, and the tree highest concentrations of epoxiconazole (Figure 9). Regarding effects on progesterone synthesis, the general picture was a stimulating effect, but at the highest concentrations of all fungicides the stimulation was decreased (Figure 9). Cytotoxicity was not found at any concentrations using the resazurin-test.







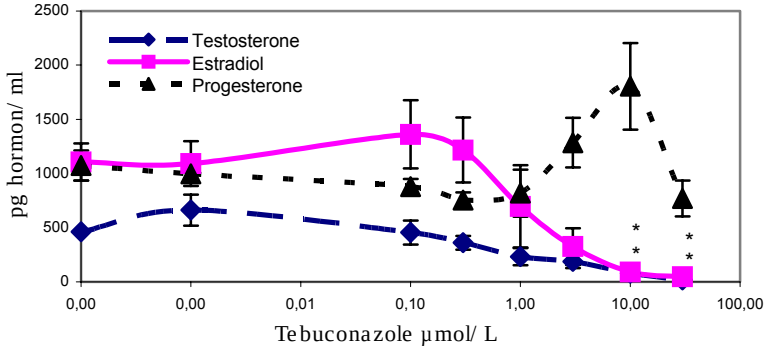


Figure 9 - *In vitro* effects of the fungicides on testosterone, progesterone and estradiol formation in human adrenocortical carcinoma cells (H295R). Data represent the mean±SEM for two to three independent experiments. E2 = estradiol. * Statistically significantly different from control (P<0.05).

3.1.6 Thyroid effects of azole fungicides

The effect on proliferation of GH3 rat pituitary tumor cells *in vitro* by T_3 and the fungicides is illustrated in Figure 10 and 11, respectively. T_3 dose-dependently stimulated the cell proliferation at 0.05 nM (LOEC) and maximally at 1.5 nM (MOEC) (Figure 10). The half maximum response (EC_{50}) of T_3 was determined at 0.20 nM. This assay was established as part of this project and the fact that we get a nice dose-response curve for T_3 verifies that the assay works.

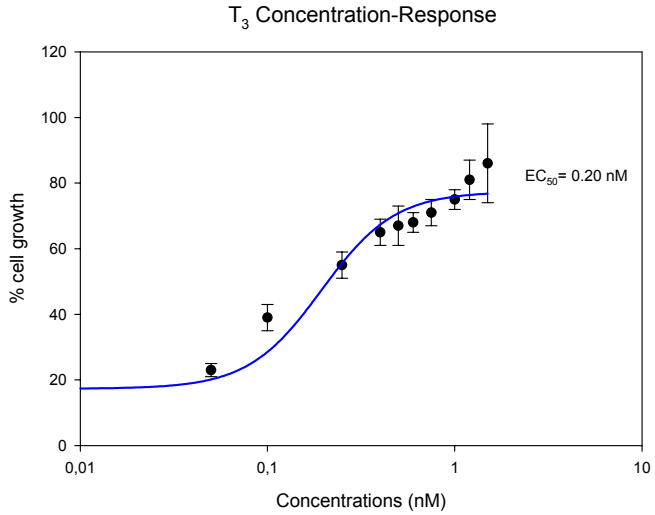


Figure 10 - Effect of T_3 on proliferation of GH3 cells. Solvent control (0.1% DMSO) was set to 0 %.
EC_{50} determined to 0.20 nM. Data represent mean±SD for three independent experiments.

Propiconazole and tebuconazole had a weak inhibitory effect on GH3 cell growth (Figure 11). At concentrations of 3 µM to 30 µM tebuconazole statistically significantly decreased the cell proliferation to approximately 70% of basal level and propiconazole significantly decreased the cell proliferation at 30 µM to approximately 80% of solvent control.

Tebuconazole significantly inhibited the T_3-induced growth of the GH3 cells at 10 and 30 µM, and for the three other pesticides a significant decreased cell growth was also seen at 30 µM (Figure 11). The decrease in cell proliferation at 30 µM, for cells treated with the fungicides alone as well as upon co-treatment 0.25 nM T3, is probably due to a cytotoxic effect, as others have demonstrated cytoxicity at these concentrations, for some of the same chemicals (Ghisari and Bonefeld-Jorgensen, 2005).

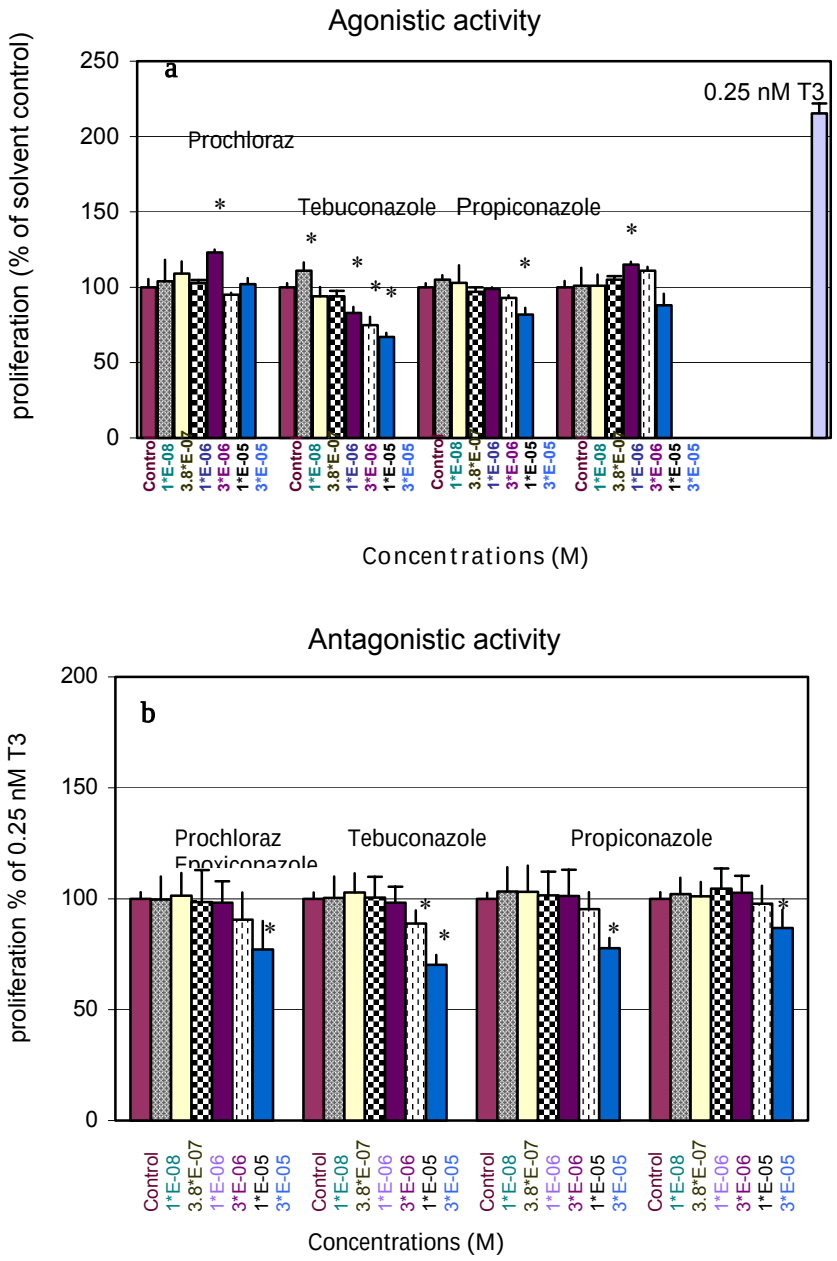


Figure 11 - Effects of the fungicides in the T-screen assay: GH3 cells treated with different concentrations of fungicides alone (a) or in the presence of 0.25 nM T3 (b). The values are given as percentage of the proliferation of solvent control (0.1% DMSO) or of positive control (0.25 nM T3). Data represent mean±SD. * Statistically significantly different ($p \leq 0.05$) from the respective controls.

3.2 *In vivo* effects

A Hershberger test and a developmental toxicity study were conducted to investigate *in vivo* effects of the azole fungicides.

3.2.1 Anti-androgenic effects in the Hershberger test

Propiconazole and tebuconazole were tested in castrated testosterone-treated rats in order to determine if the compounds were able to block androgen receptors or affect androgen levels *in vivo*. They were administered orally at

doses of 50, 100, and 150 mg/kg together with a *sc* injection of a fixed dose of testosterone. The anti-androgen flutamide was given as a positive control.

In vivo, body weights, paired kidney weights, thyroid and pituitary weights were unaffected by the treatments, whereas liver weights were increased with both fungicides. The increase in liver weights was statistically significant at the highest dose of tebuconazole and the two highest doses of propiconazole.

Compared to intact males of the same age, castrated vehicle-exposed rats had significantly reduced weights of all the reproductive organs and increased levels of LH and FSH (Table 6 and 7). The serum levels of T_4 were unaffected by castration. On the gene expression level both PBP C3 and ODC mRNA were reduced by castration, whereas TRPM-2 and Compl. C3 mRNA were increased (Table 8). All these results were in accordance with predictions based on theoretical knowledge and previous experiments.

Flutamide exerted the expected effects as well, and caused qualitatively the same effects as seen after castration. The weights of all reproductive organs were decreased; serum LH and FSH levels were increased and at gene level both PBP C3 and ODC mRNA were reduced, whereas TRPM-2 and Compl. C3 mRNA were increased. Overall it can be concluded that this Hershberger test was performed as expected.

The fungicides had no effect on neither reproductive organ weights or on hormone levels, except for the highest dose of propiconazole, for which there was a significant increase in FSH. On the gene expression level, the only significant effect of the fungicides was a decrease in the level of ODC mRNA in prostate, with all doses of propiconazole and with the highest dose of tebuconazole. This gene is regulated by androgens but is also regulated via other pathways, so in the light of the lack of anti-androgenic effects on other genes, it is conceivable that this effect is due to a non-androgen dependent effect.

In conclusion neither propiconazole nor tebuconazole had any androgen receptor blocking effects *in vivo* in the Hershberger test at doses below 150 mg/kg bw/day. This is in contrast to the imidazole fungicide prochloraz that induced anti-androgenic effects at doses between 50 and 150 mg/kg bw/day.

Table 6 - Body and Organ weights of young male rats in the Hershberger test.

	Intact animals	Castrated control	Control	Flutamide	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Tebuconazole 150 mg/kg	Propiconazole 50 mg/kg	Propiconazole 100 mg/kg	Propiconazole 150 mg/kg
Body weight (g)	183±14	166±3	165±10	165±18	164±16	165±14	170±15	170±15	171±21	173±10
Prostate (mg)	87.5±10.8*	5.7±3.6*	49.2±18.0	11.1±5.9* [a]	57.2±13.6	51.4±10.8	61.0±8.8 [a]	53.8±15.2	43.0±13.7	59.2±12.9
Seminal vesicles (mg)	114.0±36.7	12.5±3.0*	95.9±39.7	20.1±3.9*	106.2±12.4	90.5±7.7	101.4±11.1	92.4±13.3	96.5±20.1	88.5±22.4
LABC (mg)	229±24	93±11*	199±12	124±24*	202±38	216.7±22.6	222±27	208.9±25	204±38	221±23
Bulbourethral gl. (mg)	15.8±4.1*	1.7±0.7* [a]	9.0±2.2	1.8±0.8*	10.5±1.3	10.4±1.3	10.7±2.0	10.2±1.7	9.2±1.9 [a]	10.9±1.5
Pituitary (mg)	6.2±0.5	7.6±1.0	6.4±0.6	6.6±1.9	6.5±0.7	6.7±0.9	7.5±1.0	6.9±0.6	7.4±0.7	7.5±0.7
Thyroid (mg)	9.5±3.8	9.1±5.0	8.0±2.0	8.3±1.1	8.0±1.0	8.2±1.8	8.4±3.0	8.1±1.8	7.7±2.7	7.3±1.4
Liver (g)	8.2±0.6*	7.2±0.3	6.9±0.5	7.7±0.9	7.2±0.8	7.4±0.7	8.5±1.0*	7.9±0.9	8.2±1.1*	8.5±0.5*
Kidney (g)	1.5±0.1 [a]	1.3±0.1	1.3±0.1	1.3±0.1	1.2±0.1	1.3±0.1	1.4±0.2	1.4±0.1	1.4±0.2	1.4±0.1

All animals were castrated except for the intact control. All animals received testosterone propionate, except the intact and castrated controls, which only were given vehicle. The group named "Control" is the group used as control in the statistical analysis. Data represent mean±SEM; N=6 ; [a]: N= 5. * indicate statistically significant compared to controls by Dunnett's test (P<0.05).

Table 7 - Serum hormone levels of young male rats in the Hershberger test.

	Intact animals	Castrated control	Control	Flutamide	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Tebuconazole 150 mg/kg	Propiconazole 50 mg/kg	Propiconazole 100 mg/kg	Propiconazole 150 mg/kg
LH (ng/ml)	1.20±0.54	22.02±18.92*	0.82±1.34	15.79±6.17*	0.86±0.77	0.50±0.62	1.02±0.95	1.63±1.23	1.49±2.77	1.86±1.91
FSH (ng/ml)	9.80±0.97 *	32.2±5.58*	20.81±3.21	36.85±4.96*	24.23±3.70	19.74±5.75	21.60±3.54	21.51±7.83	24.07±6.90	32.71±10.8*
T_4 (nM)	127±41	110±28	140±41	123±23	145±33	141±39	146±42	122±29	116±29	127±29

All animals, except the intact and castrated controls, received testosterone propionate. The group named "Control" is the group used as control in the statistical analysis.
Data represent mean±SEM; N=6.
* indicate statistically significant compared to controls by Dunnett's test (P<0.05).

Table 8 - Gene expression in ventral prostate of young male rats in the Hershberger test.

	Intact animals	Castrated control	Control	Flutamide	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Tebuconazole 150 mg/kg	Propiconazole 50 mg/kg	Propiconazole 100 mg/kg	Propiconazole 150 mg/kg
PBP C3/ 18S	61.8±4.5	1.04±0.81*	51.7±4.66	6.07±1.85*	41.1±1.95	42.0±6.16	38.2±2.49	36.5±5.20	30.6±1.04	32.8±2.33
ODC/ 18S (x 10^{-3})	13.3±1.02*	4.42±1.38*	24.7±1.91	9.03±1.78*	24.0±1.53	22.8±1.84	17.8±0.44*	18.2±1.51*	16.6±1.30*	18.8±1.49*
TRPM-2/ 18S (x 10^{-2})	1.62±0.48	25.5±10.2*	0.93±0.08	7.56±4.13*	1.02±0.10	1.05±0.16	1.07±0.09	0.94±0.09	1.06±0.12	1.05±0.07
Compl.C3/ 18S (x 10^{-4})	4.02±1.43	19.5±9.29*	3.72±0.52	35.5±18.3*	5.71±1.62	4.97±0.85	6.87±2.59	5.40±1.52	5.23±2.61	2.40±0.19

All animals, except the intact and castrated controls, received testosterone propionate. The group named “Control” is the group used as control in the statistical analysis.
Data represent the mean±SEM; N=6.
* indicate statistically significant compared to controls by Dunnett’s test ($P<0.05$).

3.2.2 Effects on offspring after prenatal exposure

Developmental effects of epoxiconazole and tebuconazole were investigated by dosing pregnant dams with test compounds and examination of their fetuses and offspring for effects on sexual differentiation.

3.2.2.1 Effects on GD 21 fetuses

As seen from Table 9 the highest dose of tebuconazole lead to a decreased body weight gain in dams, probably due to effects on both the dam and the uterine content, increased loss of fetuses and decreased fetal weight on GD 21. The highest dose of epoxiconazole caused increased loss of fetuses and decreased fetal weight in the absence of effects on maternal weight gain. Many of the dead fetuses (27 of 128) had died very late in the gestation period, while such late fetal death was not seen in the controls (0 of 70). The lower dose of epoxiconazole also seemed to induce increased fetal death, but the differences were not statistically significant. Very late fetal death occurred in 4 out of 92 cases compared to 0 in the controls. Although not statistically significant these findings raise concern for effects on fetal survival also at this dose level.

3.2.2.2 Immunohistochemistry

StAR and P450scc immunostaining were comparable to controls. However, it cannot be excluded that effects on PBR and 17β HSD immunostaining were present, but due to lack of control foetuses for this analysis, no conclusions could be made.

Table 9 - Pregnancy and litter data for the developmental study.

	Control	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Epoxiconazole 15 mg/kg	Epoxiconazole 50 mg/kg
Dams and litters					
No. of dams (viable litters)	N=13 (13)	N=12 (12)	N=10 (8) ¤	N=10 (9)	N=7 (1-2) ¤
BW gain. GD7-GD21 (g)	85.38 ± 11.9	77.17 ± 14.4	**61.00±12.5***	87.70 ± 16.1	92.14 ± 14.0
BW gain. GD7-PND1 (g)	20.62 ± 7.2	17.58 ± 6.8	**13.50 ± 10.6***	18.11 ± 6.7	15.00 ± 8.5
BW gain PND1-PND13 (g)	7.53 ± 16.3	7.75 ± 16.84	-4.57 ± 12.9	-6.89 ± 24.8	*20*
Gestation length (d)	22.46 ± 0.5	22.67 ± 0.5	**23.40 ± 1.2****	22.67 ± 0.7	**23.71 ± 0.8****
% postimplantation loss	6.55 ± 5.1	10.31 ± 11.2	**27.32 ± 23.5***	16.01 ± 30.0	**34.25 ± 18.2***
% perinatal loss	9.67 ± 8.0	13.37 ± 12.5	**54.97 ± 36.9****	18.21 ± 30.0	**88.78 ± 29.7****
Litter size	11.15 ± 1.7	10.75 ± 3.6	8.75 ± 3.8	9.90 ± 4.1	8.67 ± 3.1
Born alive per litter	10.92 ± 1.7	10.67 ± 3.7	8.38 ± 3.8	9.80 ± 4.0	**4.33 ± 5.9****
Born dead per litter	0.23 ± 0.4	0.08 ± 0.3	0.37 ± 0.7	0.10 ± 0.3	**4.33 ± 2.9****
% PN death	3.39 ± 5.55	3.36 ± 7.04	**27.00 ± 37.5***	2.78 ± 5.9	**69.44 ± 52.9****
% males	44.76 ±17.6	56.42 ± 11.7	40.36 ± 18.6	45.45 ± 13.13	34.72 ± 33.42
Offspring (data from viable litters)					
Birth weight (g)	5.53 ± 0.3	5.64 ± 0.5	5.63 ± 0.8	**6.21 ± 0.6****	6.36 ± 0.3
BW. PND 13 (g)	23.25 ± 2.6	21.59 ± 4.14	22.39 ± 5.02	21.48 ± 4.8	*23.4*
Male AGD (mm)	3.41 ± 0.2	3.39 ± 0.1	3.51 ± 0.2	**3.65 ± 0.22***	3.41 ± 0.3
Male AGD/cuberoot bw	1.92 ± 0.1	1.90 ± 0.1	1.96 ± 0.1	1.96 ± 0.1	1.83 ± 0.2
Female AGD (mm)	1.72 ± 0.1	1.80 ± 0.1	**1.91 ± 0.1***	**1.95 ± 0.2****	*1.71*
Female AGD/cuberoot bw	0.98 ± 0.03	1.02 ± 0.06	**1.09 ± 0.07***	**1.08 ± 0.08***	*0.96*
No. aerolas in males	2.08 ± 0.6	**3.43 ± 0.9****	**3.07 ± 2.5****	2.53 ± 1.1	*3.38*
No. aerolas in females	12.5 ± 0.4	12.46 ± 0.4	12.31 ± 0.4	12.32 ± 0.2	*12*
GD21 Caesarian section ¤					
No. of dams	N=6	N=7	N=8+2 ¤	N=9	N=14+4 ¤
Maternal bw (g)	307.17 ± 22.4	297.00 ± 27.2	**281.00 ± 26.5***	287.33 ± 29.8	285.73 ± 17.7
Adjusted bw (g)	232.70 ± 14.9	234.00 ± 16.8	223.31 ± 20.4	231.49 ± 15.9	223.13 ± 21.0
No. implantations	12.50 ± 2.1	12.00 ± 3.2	11.60 ± 1.5	10.22 ± 4.3	12.06 ± 2.4
No. fetuses	11.67 ± 2.1	11.14 ± 3.6	9.40 ± 2.1	9.11 ± 4.9	9.00 ± 4.1
% postimplantation loss	6.45 ± 7.9	9.10 ± 11.3	**21.54 ± 9.4***	20.87 ± 33.5	**28.14 ± 26.9***
% late res	1.28 ± 3.1	2.38 ± 6.3	6.14 ± 4.2	13.89 ± 33.3	**24.88 ± 27.3***
% very late res	0.0 ± 0.0	2.38 ± 6.3	2.39 ± 4.2	4.16 ± 11.8	**15.12 ± 24.0***
% males	56.01 ± 17.2	46.22 ± 20.9	49.74 ± 20.6	46.41 ± 18.8	53.57 ± 25.3
Fetal weight male (g)	4.45 ± 0.3	3.84 ± 0.7	**3.44 ± 0.9****	3.98 ± 0.9	**3.79 ± 0.7***
Fetal weight female (g)	4.18 ± 0.4	3.61 ± 0.6	**3.40 ± 0.9***	3.83 ± 0.8	**3.54 ± 0.7***
No. litters for AGD #	N=3	N=4	N=4	N=6	N=10
Male AGD (mm)	3.39 ± 0.3	3.50 ± 0.03	3.29 ± 0.4	3.54 ± 0.1	3.40 ± 0.1
Male AGD /cuberoot bw	2.08 ± 0.1	2.25 ± 0.2	**2.30 ± 0.1***	**2.31 ± 0.1***	2.25 ± 0.1
Female AGD (mm)	1.65 ± 0.1	**1.87 ± 0.2***	**2.02 ± 0.1****	**1.91 ± 0.3****	**1.92 ± 0.1****
Female AGD/cuberoot bw	1.04 ± 0.1	1.23 ± 0.2	**1.43 ± 0.2****	**1.28 ± 0.2***	**1.29 ± 0.1****

Data represent group means based on litter means±SD.
PN = postnatal. Res = resorption i.e. regression of the fetus;
* or ** statistically significant compared to control ($p<0.05$ or $p<0.01$, respectively). Significant values are indicated in bold.
¤ Because of problems with parturition caesarian section (CS)(GD23-25) was performed on 2 dams in the 100 mg/kg tebuconazole group and 4 dams in the 50 mg/kg epoxiconazole group. These data were included in the analysis of GD21 CS data.
AGDs were only measured in the 2nd set of animals.
Values written in Italic are from only one dam/litter why no SD are shown.

3.2.2.3 Hormone analysis

In fetuses tebuconazole caused a statistically significant increase in testicular 17α-hydroxy-progesterone and progesterone levels, and a decrease in

testosterone levels. Epoxiconazole had no significant effect on the measured hormone levels in fetuses (Table 10).

Table 10 - Testicular hormone concentrations in male fetuses at GD21.

	17α-hydroxyprogesterone (pg/testis)	Testosterone (ng/testis)	Progesterone (ng/testis)	Testosterone production (ng/testis)	Progesterone production (ng/testis)
Control	1.95±0.54(4)	1.75±0.71(5)	0.037±0.025(5)	3.95±1.71(6)	0.02±0.01(6)
Tebuconazole 50 mg/kg	**8.39±2.59*** 7)	1.25±0.40(7)	**0.103±0.035*** (7)	4.77±3.49(6)	0.29±0.62(6)
Tebuconazole 100 mg/kg	**6.59±3.88*** (9)	**0.88±0.46*** (9)	0.084±0.063(9)	3.34±2.59(5)	0.04±0.03(5)
Epoxiconazole 15 mg/kg	1.76±1.36(6)	1.62±0.59(8)	0.029±0.019(8)	4.41±3.27(6)	0.00±0.00(6)
Epoxiconazole 50 mg/kg	0.94±0.48(13)	1.11±0.56(20)	0.027±0.019(20)	4.00±3.27(10)	0.00±0.01(10)

Fetal testes were extracted with diethylether before measurement of progesterone, 17α-progesterone and testosterone. Other fetal testes were incubated in a water bath at 37°C for 3 hrs before measurement of testosterone and progesterone in supernatants. Data represent the mean±SD; ( ) = n; * statistically significantly different compared to control (P<0.05).

In plasma from mothers obtained at GD 21, the highest dose of tebuconazole led to a marked increase in the progesterone level (7-fold increase), and a significant decrease in T_3. Epoxiconazole led to an increase in the progesterone (7-fold increase) and surprisingly also to a pronounced increase in the testosterone level (2-fold), at the highest dose (Table 11). The increased progesterone levels in the mothers could be the explanation for the increased gestational length (Table 9).

Table 11 - Maternal plasma concentrations of hormones and lipids at GD 21.

	Cholesterol Mean (mM)	Triglyceride Mean (mM)	T_3 Mean (nM)	T_4 Mean (nM)	Testosterone Mean (nM)	Progesterone Mean (nM)
Control	1.85±0.28(6)	7.44±2.26(6)	2.38±0.48(7)	53.53±17.45(7)	0.40±0.25(6)	48±32(6)
Tebuconazole 50 mg/kg	1.68±0.22(7)	4.01±0.94(7)	2.36±0.24(7)	53.14±11.95(7)	0.22±0.09(7)	113±114(7)
Tebuconazole 100 mg/kg	2.20±0.30(8)	6.23±1.71(8)	**1.99±0.23*** (8)	38.26±10.74(8)	0.32±0.17(8)	**354±163***(8)
Epoxiconazole 15 mg/kg	1.90±0.31(8)	6.14±3.92(8)	2.47±0.25(8)	46.44±9.05(8)	0.49±0.31(9)	170±141(9)
Epoxiconazole 50 mg/kg	2.31±0.74(14)	12.07±7.45(14)	2.29±0.23(14)	44.09±13.92(14)	**0.82±0.43*** (15)	**349±190*** 14)

Data represent the mean±SD; ( ) = n; * statistically significantly different compared to control (P<0.05).

3.2.2.4 Gene expression levels

Table 12 - Overview of gene expression in testis from male fetuses (GD 21).

	Control	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Epoxiconazole 15 mg/kg	Epoxiconazole 50 mg/kg
ScarB1 mRNA	↔	↔	↔	↔	↔
P450scc mRNA	↔	↔	↔	↔	↔
P450c17 mRNA	↔	↔	↔	↔	↔
StAR mRNA	↔	↔	↔	↔	↑

The selected genes are all involved in steroidogenesis according to Figure 3. The level of gene expression.
relative to the housekeeping gene 18S.
↔ = no effect. ↑ = up regulated

Regarding expression levels of genes involved in testicular testosterone production no effects were observed except for an induced expression of the StAR mRNA in fetal testis for the highest dose of epoxiconazole (P<0.026).

3.2.2.5 Endpoints related to PND 1, PND 13 and PND 16 Pups

As seen from Table 9, the highest doses of tebuconazole and epoxiconazole led to an increased length of gestation, increased loss of fetuses and a marked increase in postnatal death of the pups. In addition, the highest dose of epoxiconazole induced a high frequency of stillbirth leading to marked reduction in live litter size. The high doses of both chemicals induced slightly decreased body weight gain in the dams during pregnancy, but the effect was only statistically significant for the high dose of tebuconazole.

3.2.2.6 Anogenital distance and nipple retention

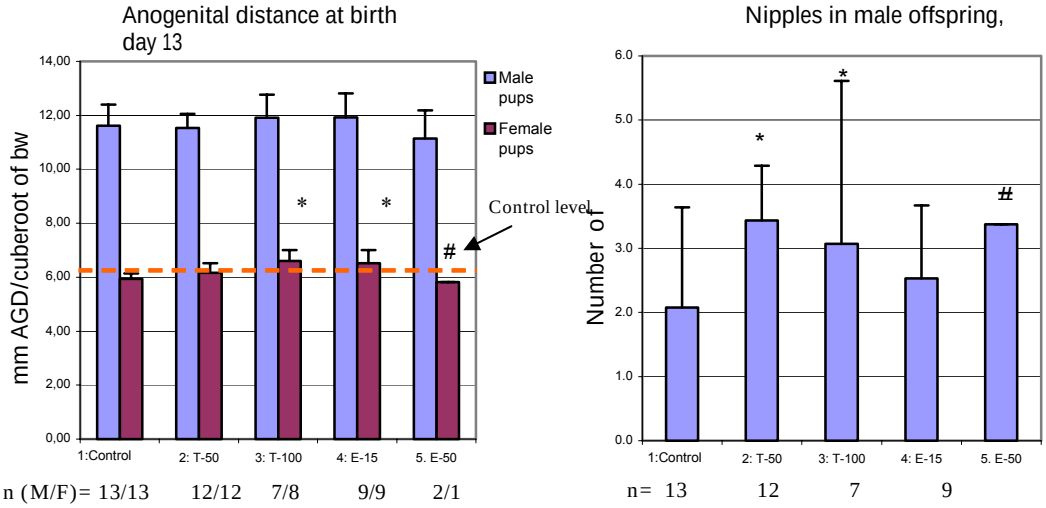


Figure 12 - Effect on anogenital distance at birth and nipple retention PND 13, caused by perinatal exposure to epoxiconazole or tebuconazole. AGD is shown as distance per cuberoot of body weight. T-50 and T-100 = tebuconazole (50 and 100 mg/kg bw/day). E-15 and E-50 = epoxiconazole (15 and 50 mg/kg bw/day). The data represent the mean±SD. * statistically significant compared to control. **#** Only one pup.

Both tebuconazole and epoxiconazole increased the anogenital distance in female offspring at birth. This was statistically significant for the highest dose of tebuconazole and the lowest dose of epoxiconazole (Figure 12 and Table 9). In the male offspring the anogenital distance is significantly increased in the lowest epoxiconazole dose group before adjusting for body weight (Table 9)**,** but when AGD is divided by the cubic-root of the body weight as represented in Figure 12 there is no increase. In the fetuses (GD21) tebuconazole and epoxiconazole increased the body weight-adjusted anogenital distance in both males and females (Table 9). Furthermore, both doses of tebuconazole resulted in an increased number of nipples in male pups at PND 13, a tendency that was also seen for epoxiconazole 15 mg/kg bw/day, but was not significant (Figure 12 and Table 9).

3.2.2.7 Hormone analysis

As an increased AGD was seen in the female pups at birth (Figure 12), it was of interest to analyze estradiol in ovaries and testosterone in male plasma. A clear tendency towards lowered estradiol levels in female pups PND 16 was seen for both compounds. Also the plasma testosterone in male pups at the highest dose of tebuconazole and both doses of epoxiconazole seem to be lowered, however, the effects were not statistically significant (Table 13).

Table 13 - Hormone concentrations in ovaries (females) and testis (male) from pups PND 16.

	Females Estradiol in ovaries Mean(pg/ovary)	Males Testosterone in plasma Mean (ng/ml plasma)
Control	8.40±3.90(13)	0.14±0.18(12)
Tebuconazole 50 mg/kg	8.40±7.00(12)	0.17±0.18(12)
Tebuconazole 100 mg/kg	5.60±4.60(7)	0.11±0.09(7)
Epoxiconazole 15 mg/kg	5.00±2.70(9)	0.07±0.14(7)
Epoxiconazole 50 mg/kg	*3.6 (1)*	*0.02 (1)*

Data represent the mean±SD. ( ) = n. Values written in italic are from one animal only.

3.2.2.8 Gene expression levels

No significant effects were seen on expression levels of genes in the prostate or epididymis in male pups PND 16 (Table 14).

Table 14 - Changes in mRNA expression levels in prostate and epididymis PND 16. All data are from prostates except for PEM mRNA which is from epididymis.

	Control	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Epoxiconazole 15 mg/kg	Epoxiconazole 50 mg/kg
AR	↔	↔	↔	↔	↔
ODC	↔	↔	↔	↔	↔
Compl.C3	↔	↔	↔	↔	↔
IGF-1	↔	↔	↔	↔	↔
PBPC3	↔	↔	↔	↔	↔
TRPM-2	↔	↔	↔	↔	↔
PEM	↔	↔	↔	↔	↔

The level of gene expression relative to the housekeeping gene 18S rRNA.
↔ = no effect.

3.2.2.9 Autopsy, Organ weight and Histopathology

No histopathological effects on the male genital tubercle were observed. Body and organ weights of male and female rat offspring at PND 16 are shown in Table 15.A statistically significant increased liver weight is observed at a dose of 100 mg/kg bw/day tebuconazole. For epoxiconazole at 50 mg/kg bw/day a tendency towards an increase in liver weight was seen as well. No effect on the reproductive organ weights was observed for tebuconazole. Epoxiconazole was associated with increased weights of ventral prostate. The data is however based on only 2 organs, and no conclusions can therefore be drawn.

Female reproductive organs were unaffected, and in the haematoxylin and eosin stain, no histopathological effects were observed in any of the examined male organs (testes and prostates).

Table 15 - Effects of epoxiconazole (E) and tebuconazole (T) on male and female pups PND 16.

Male	Control	Tebuconazole 50 mg/kg	Tebuconazole 100 mg/kg	Epoxiconazole 15 mg/kg	Epoxiconazole 50 mg/kg
Body weights (g) §	28.9±0.4(42)	26.6±0.7(46)	27.9±1.1(25)	25.4±o.8(29)	29.3±0.7(6)
Right testis (mg) §	54.7±0.7(43)	53.0±1.7(44)	56.6±2.8(25)	50.2±1.7(29)	67.0±2.6(6)
Left testis (mg) §	54.4±0.9(43)	52.7±1.7(44)	54.5±2.9(25)	49.8±1.9(29)	67.8±2.2(6)
Epididymides (mg)§	20.8±0.5(25)	20.1±0.7(23)	21.4±1.1(14)	20.0±1.2(16)	23.3±0.9(2)
Ventral prostate (mg)§	12.4±0.4(25)	12.5±0.8(21)	13.2±1.1(14)	12.4±0.9(16)	21.2±1.6*[a]
Seminal vesicles (mg)§	8.7±0.5(25)	8.3±0.5(23)	9.4±0.9(13)	7.2±0.4(16)	9.4±1.8(2)
LABC (mg)§	26.0±0.1(12)	22.3±1.4(10)	25.3±1.8(7)	24.1±0.6(7)	*30.6*
Bulbourethral gl. (mg)	1.6±0.1(11)	1.5±0.1(11)	1.7±0.2(7)	1.7±0.1(8)	*2.7*
Thyroid (mg)§	3.6±0.2(31)	3.8±0.2(33)	3.6±0.2(18)	3.3±0.2(24)	3.0±0.3(3)
Adrenals (mg) §	8.5±0.3(26)	7.9±0.5(24)	8.7±0.9(14)	7.2±0.4(16)	6.1±3.3(2)
Kidneys (mg)§	295.5±7.9(13)	272.2±18.4(12)	295.3±27.5(7)	259.8±18.9(8)	*333.4*
Liver (mg)§	735.6±17.8(25)	702.3±40.6(24)	795.2±49.9*(14)	602.8±39.8(16)	801.7±58.6(2)
Female					
Body weights (g)	29.0±0.7(24)	26.5±1.2(21)	28.2±1.9(15)	27.3±1.6(15)	27.3±0.3(2)
Thyroid (mg)	3.9±0.6(12)	4.4±0.3(11)	3.9±0.5(6)	3.4±0.4(8)	*2.6*
Uterus (mg)§	18.7±0.6(13)	19.1±1.3(12)	19.7±1.5(6)	18.3±1.2(9)	*16.6*
Ovaries (mg)	5.5±0.3(9)	5.1±0.3(7)	5.7±0.4(5)	4.7±0.3(7)	*7.0*

Data represent least squares means±SEM. ( ) = n;
* Statistical significant different compared to controls ($p<0.05$). § Statistically significant effect of the covariate, body weight ($p<0.05$).
[a] only two organs were included in this analysis. *Values written in italic are from only one organ why no SEM is shown*.

3.2.2.10 Semen quality analysis PND 224

Semen quality was analyzed only in the low dose groups due to maternal or fetotoxic effects in the high dose groups. The semen quality analysis showed that three samples out of twelve animals from the group exposed to the low dose of epoxiconazole did not contain any sperm cells, while all other samples contained 200-400 sperm cells (11 controls, 11 tebuconazole, 9 epoxiconazole). The difference is statistically significant (p=3.7%) when compared to the combined results from the control group and the tebuconazole group, but not when compared to the control group alone.

The results of the semen quality analysis for animals with sperm cells are shown in Table 16. Statistical analysis of the sperm motility including or excluding the three animals without sperm cells did not show any significant differences.

Table 16 - Semen quality analysis – motility.

Semen quality	Control	Tebuconazole 50 mg/kg	Epoxiconazole 15 mg/kg#
Number of animals	11	11	9
Sperm cells used for the analysis	292±18	263±14	271±18
Motile cells, count	143±19	153±14	143±15
Percent motile	48.6±5.9	57.6±3.7	53.0±4.1
Progressive cells, count	73±12	80±9	70±9
Percent progressive	24.9±3.5	30.4±3.1	26.1±3.1

Data represent means±SEM. T-50 = tebuconazole 50 mg/kg bw/day.
Without three animals not having any sperm cells.

No effects were seen on curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), or progression parameters such as average path velocity (VAP), straight-line velocity (VSL) or straightness (STR) (data not shown).

3.3 Overview of results

Table 17 shows an overview of the qualitative results obtained from this study. Some of the results for prochloraz are from previous studies.

Table 17 - An overview of the most important observations for the azole fungicides in the in vitro and in vivo test systems.

	Epoxiconazole	Prochloraz	Propiconazole	Tebuconazole
***In Vitro* assays**				
Estrogenic activity	+	-	+	-
Anti-estrogenic effect	+	+	+	+
Anti-androgenic effect	+	+	+	+
Aromatase inhibition	+	+	+	+
AhR activity	+	+++	+	+
Testosterone production	↓	↓	↓	↓
Progesterone production	↑	↑	↑	↑
E2 production	↓	↓	↓	↓
Thyroid effect	-	-	-	antagonism
***In Vivo* experiments**				
Hershberger Study	Not investigated	+++	↑ liver weights ↑ FSH level ↓ ODC mRNA	↑ liver weights ↓ ODC mRNA
Developmental Toxicity			Not investigated	
Gestational length	↑	↑	-	↑
Fetal toxicity	50 mg/kg	> 100 mg/kg	-	10 mg/kg
AGD/ cuberoot bw in males		↓	-	
AGD/cuberoot bw in females	↑	↑	-	↑
Nipples (male PND 13)	-	↑	-	↑
Hormone analysis in fetal testis	-	↑ Progesterone ↓ Testosterone	-	↑ Progesterone ↓ Testosterone
Hormone levels in mothers	↑ Testosterone ↑ Progesterone		-	↑ Progesterone ↓ T_3
Gene expression (PND 16 prostate)	-	↓ ODC, PBP C3 and IGF-1 mRNA	-	-

4 Discussion

Several pesticides have been demonstrated to be ER agonists or AR antagonists in various *in vitro* test systems (Andersen *et al.*, 2002; Kojima *et al.*, 2004). However, the affinities for the steroid receptors are very low relative to endogenous hormones and therefore it could be questioned whether these mechanisms alone can induce effects *in vivo* at environmentally relevant concentrations. However, it is conceivable that chemicals with the ability to act via several endocrine pathways are more likely to cause effects *in vivo* due to the combined response from different mechanisms (Sanderson, 2006; Sharpe, 2006). Furthermore, as humans are rarely, if ever, exposed to one single chemical at a time, the combined exposure to several chemicals acting via the same pathway may cause adverse effects in humans.

The results from the *in vitro* investigations in this study demonstrate that the three azole fungicides: propiconazole, tebuconazole, and epoxiconazole all had the ability, like prochloraz, to act via several different mechanisms. The three triazoles were less potent than prochloraz to act as ER antagonists (prochloraz > tebuconazole ≈ epoxiconazole ≈ propiconazole) and AhR agonists (prochloraz >>> tebuconazole ≈ epoxiconazole ≈ propiconazole) while the potency as AR antagonists was similar. Like prochloraz, the triazoles were aromatase inhibitors, although again, with lower potency than prochloraz (prochloraz >epoxiconazole ≈ tebuconazole > propiconazole).

While several pesticides have been reported to be ER agonists *in vitro* only prochloraz (Andersen *et al.*, 2002) and some pyrethroid insecticides (Kim *et al.*, 2004) have, to our knowledge, been reported to be ER antagonists. The impact of this mechanism for the integrated response *in vivo* is unknown at present and no standardized method for investigating this effect *in vivo* have been established. The results obtained in this study on aromatase (CYP19) inhibition is in accordance with previous studies (Trosken *et al.*, 2004;Trosken *et al.*, 2006;Vinggaard *et al.*, 2000) in which prochloraz was also identified as a stronger aromatase inhibitor than the other fungicides tested.

The higher potency of prochloraz towards the ER, and especially the AhR, indicates that the molecular structure of this compound favour the ability to bind to these receptors. In general, high-affinity AhR ligands are planar aromatic polycyclic molecules such as the prototypical ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Rifkind, 2006). The number and places of halogens affect the planarity of the molecule and computer modelling could be used for comparison of the conformation of the different azole molecules but such analysis have not been performed. Since the affinities of the triazoles for the ER and AhR were similar in spite of their varied number and placement of halogens, it seems more likely that the differences in affinities between prochloraz and the triazoles are related to the imidazole structure.

The results from the H295R steroid hormone synthesis assay showed that the triazoles, as well as prochloraz, inhibited testosterone and estradiol

production, and increased progesterone production. This indicates that enzymes involved in the conversion of progesterone to testosterone are inhibited. Azole fungicides and azole antifungal drugs are designed to inhibit the biosynthesis of ergosterol in fungi by inhibiting the cytochrome P450 enzyme 14α-lanosterol demethylase (CYP51). In fungi the lack of ergosterol leads to collapse of the cell membrane. In humans CYP51 is important for the conversion of lanosterol to cholesterol and in the production of meiosis activating steroids. Thus, inhibition of human CYP51 may disturb steroid synthesis. Prochloraz, propiconazol, tebuconazol, and epoxiconazol have all been reported to inhibit human CYP51 (Trosken *et al.*, 2006) but with lower potency than the antifungal drugs miconazole and ketoconazole. The azole compounds are not selective for CYP51 and a range of other cytochrome P450 enzymes, including aromatase and other CYP enzymes involved in steroidgenesis, are also affected by azole compounds. Hence, CYP17 (17α-hydroxylase and/or 17,20-lyase activity), which are involved in the conversion of cholesterol to testosterone (see Figure 3), has been demonstrated to be inhibited by several azole compounds including the imidazole fungicides imazalil and prochloraz as well as and some imidazole antifungal drugs (e.g., ketoconazol) (Ayub and Levell, 1987;Ayub and Levell, 1989;Mason *et al.*, 1987). Hence, the reduced testosterone and estradiol synthesis and increased progesterone production observed for all four azole fungicides in the H295R steroid synthesis assay might, at least partly, be due to inhibition of CYP17.

Several other cytochrom P450 enzymes have also been reported to be affected by azole compounds. Enzymes of the CYP1, CYP2 and CYP3 gene families were induced by some azoles (Juberg *et al.*, 2006;Mason *et al.*, 1987;Sanderson *et al.*, 2002;Sun *et al.*, 2005) and inhibited by others (Zhang *et al.*, 2002).The triazole fungicide, fenbucazole, was recently reported to be a phenobarbital-type inducer of mouse liver cytochrom P450 and to cause a dose-dependent increase in liver weight associated with hepatocellular hypertrophy and vacuolation (Juberg *et al.*, 2006). In a recent study in adult male rats, the four triazole fungicides: fluconazole, myclobutanil, propiconazole, and triadimefon all affected the expression of several CYP genes in liver and testis including genes involved in steroid metabolism (Tully *et al.*, 2006). The induction of CYP1 enzymes is mediated by the Ah receptor. The Ah receptor is a transcription factor that after binding of ligand causes increased gene transcription of Ah receptor responsive genes such as the CYP1 gene family. CYP1A1 and 1A2 metabolize estradiol to 2- and 4-hydroxy estradiol (Rifkind, 2006) and induction of these enzymes could therefore affect the hormone concentration in the organism. All four azole compounds in this study acted as AhR agonist and hence, they might induce the activity of CYP1 enzymes. Prochloraz was by far the most potent of the four azoles.

No significant and convincing effect on thyroid-receptor mediated proliferation was found for any of the azole fungicides. However, in a similar assay prochloraz inhibited the T_3-induced growth of GH3 cells in a concentration dependent way to 55 % of the reference level (Ghisari and Bonefeld-Jorgensen, 2005) and in animals both prochloraz and tebuconazole have shown effects on thyroid function (Pesticidkontoret, 1996b;Vinggaard *et al.*, 2002). However, this effect could be due to other mechanisms than direct interaction with the TR.

The anti-androgenic effects observed in the AR assay *in vitro* were not confirmed *in vivo* when tebuconazole and propiconazole were tested in the

short-term Hershberger assay. In contrast to prochloraz that induced marked anti-androgenic effects at doses between 50 and 150 mg/kg bw/day, no anti-androgenic effects were observed for tebuconazole or epoxiconazole at doses below150 mg/kg bw/day. At 150 mg/kg bw/day, FSH levels were increased by propiconazole, and at this dose level both compounds caused a down-regulation of ODC mRNA in ventral prostates. ODC is an androgen-regulated gene but is regulated by other pathways as well including regulation by estrogens (Nellemann *et al.*, 2005). Since none of the other end points (weight of androgen dependent organs, expression of androgen responsive genes or serum concentration of LH) were affected we believe that the effects observed on ODC mRNA expression are due to a non-androgen-regulated pathway. The design of the Hershberger assay is optimised to detect effects on organ weights and not for detecting changes in gene expression and our previous experience supports that changes in weights of reproductive organs is a more sensitive parameter for anti-androgenicity in this animal model than gene expression in prostates. Liver weights were increased with the highest doses of both tebuconazol and propiconazole. A stimulation of liver growth is often observed with chemicals that induce microsomal liver enzymes including the cytochrom P450 enzyme system (Juberg *et al.*, 2006) and this has been observed previously for prochloraz.

No other studies on anti-androgenic effects of azole fungicides in adult animals were found, but among the pharmaceuticals, the imidazole ketoconazole exhibited anti-androgenic effects in rats by reducing serum androgen level and increasing estradiol, FSH and LH levels. The weights of androgen sensitive organs (epididymides, prostate, and seminal vesicles) were reduced (Marty *et al.*, 2001;O'Connor *et al.*, 2002). Ketoconazole also reduced testosterone synthesis in male human volunteers after a single oral dose of 400 mg and ketoconazole as well as other imidazole antifungal drugs (isoconazole, miconazole, econazole, and clotrimazole) inhibited testosterone production in mouse Leydig cells *in vitro* (Schurmeyer and Nieschlag, 1984). The triazole compound fluconazole did not reduce testosterone levels in men after oral doses of 25 or 50 mg/kg lgv/day for 28 days (Hanger *et al.*, 1988).

Examination of reproductive development in offspring after exposure during pregnancy and lactation is presently considered to be the most sensitive animal model system to detect endocrine disrupting effects. In mammals sex determination and sexual differentiation are under strictly hormonal control and even minor disturbances in steroid hormone concentration or function during critical periods of development may affect the process (Sharpe, 2006). In this study, effects on reproductive development in offspring (fetuses and pups) were investigated for two of the triazoles: tebuconazole and epoxiconazole. Epoxiconazole was not included in the original study plan, but during 2005 we were informed that the use of epoxiconazole had increased dramatically and now was one of the most widely used azole fungicides in Denmark. In light of the negative results for propiconazole in the Hershberger test and of the more limited use of this pesticide in Denmark, we decided to include epoxiconazole instead of propiconazole.

Previous studies have demonstrated that perinatal exposure to prochloraz feminized male offspring and virilized female offspring (Laier *et al.*, 2006; Noriega *et al.*, 2005; Vinggaard *et al.*, 2005a). The results from this study, demonstrate that also tebuconazole cause feminization of male offspring as both doses (50 and 100 mg/kg bw/day) caused increased number of nipples

and at the highest dose, the testosterone concentration in fetal testis was reduced. The AGD was unaffected at birth but in male fetuses (GD21) an increased AGD, an indicator of masculinization, was observed for the highest dose of tebuconazole. However, this effect on AGD had disappeared after birth and since the other end-points mentioned indicate feminization, our conclusion is that the dominating effect of tebuconazole in male offspring is a feminization. In female fetuses, both doses of tebuconazole caused increased AGD and, for the highest dose, this effect was also evident at birth. The virilizing effect in female offspring is also supported by a decreased estradiol concentration in the ovaries at PND16, although this effect was not statistically significant. The feminizing effect of males and virilizing effect of females are in agreement with another study demonstrating reduced weight of epididymis in adult male offspring and reduced uterus weight in adult female offspring after perinatal tebuconazole exposure (Moser *et al.*, 2001) although the weight of epididymis and uterus was not affected in the offspring at PND16 in our study.

The testicular progesterone level in male fetuses was elevated after tebuconazole exposure and this, together with the lowered testosterone level, indicates a direct impact on the steroid synthesis pathway in the Leydig cells. This is also supported by the *in vitro* results on steroid hormone synthesis obtained for tebuconazole. The effects observed on hormone concentrations and nipple retention for tebuconazole are comparable to our previous results for prochloraz (Vinggaard *et al.*, 2005a). The changes in hormone level may (as mentioned earlier) be an indication of CYP 17 inhibition.

Epoxiconazole acts differently *in vivo* than the other tested azoles, as a marked fetotoxic effect was observed. The dams dosed with 50 mg/kg were in general unable to deliver their pups. Only two litters were born normally while the other litters were included in the caesarian sections at GD21. Thus, the data for this dose of epoxiconazole on the live born pups is based on a very limited number of animals. Like tebuconazole, epoxiconazole seemed to increase AGD in male fetuses but the effect is not persistent at birth. No feminizing effects on male fetuses were seen. In contrast an increased birth weight was seen with epoxiconazole which may be related to the marked up-regulated levels of testosterone in the dams. We suggest that the increased androgen exposure during pregnancy may have had a growth promoting effect on the pups. Overall, epoxiconazole seems to alter sex hormone levels in the dams, but not in the fetuses. In female offspring, AGD was increased in fetuses as well as in pups at birth indicating a virilizing effect. As for tebuconazole, the virilizing effect of epoxiconazole is supported by a lower (non-significantly) estradiol concentration in the ovaries at PND16.

Our findings on epoxiconazole are in accordance with the studies reported when epoxiconazole was approved for use in Denmark (Pesticidkontoret, 2003). In these studies, the fertility of male rats was reduced, gestational length was increased, the number of live born rat pups was reduced, and the weight of the adrenal glands in male offspring was reduced in a 2-generation study in rats using doses between 30 ppm (approx. 3 mg/kg bw/day) and 1500 ppm (approx. 125 mg/kg bw/day) in the feed. To our knowledge, no other studies on reproductive or endocrine disrupting effects of epoxiconazole have been published.

Hence, both triazoles caused virilization of the female offspring after perinatal exposure and these effects resemble those previously observed for prochloraz

(Laier *et al.*, 2006). Male offspring was feminized by tebuconazole but not by epoxiconazole. Epoxiconazole was a clear reproductive toxicant and this effect may overshadow any possible endocrine disrupting effect in male pups.The effects induced by tebuconazole were comparable to effects induced by prochloraz (her så vi effekter ved 30 mg/kg) although prochloraz had a higher potency compared to tebuconazole.

Both tebuconazole and epoxiconazole induced a high plasma concentration of progesterone in the mothers. This is probably the reason for the increased gestational length as also previously seen for prochloraz (Vinggaard *et al.*, 2005a). *In utero* exposure to natural or synthetic progesterons can induce hypospadia in male mice and the synthetic progesterone medroxyprogesterone acetate feminize male and virilizes female genitala (Willingham *et al.*, 2006). Thus, the high maternal progesterone concentration is likely to be involved in the virilizing effect of the female offspring. Whether any potential aromatase inhibition and/or ER antagonism *in vivo* are also involved cannot be ruled out from these data. The limited hormone analyses performed on the female pups indicate a tendency towards reduced estradiol concentration in the ovary, while effects on fetal progesterone levels were not investigated. For epoxiconazole, the maternal testosterone concentration in plasma was markedly increased and this may also have contributed to the effects observed in the offspring. Besides, it indicates a virilizing effect in the mothers. Since epoxiconazole was not included in the Hershberger assay, we have no hormone measurements from adult male rats but in studies reported as a part of the approval (Pesticidkontoret, 2003) increased serum concentrations of testosterone, androstendion, FSH, and ACTH as well as reduced estradiol, corticosterone, and aldosterone was reported in adult male rats. In females increased serum concentrations of dihydroepiandrosterone, androstendion, LH, FSH, and ACTH as well as reduced estradiol, prolactin, corticosterone, and aldosterone was reported after oral administration of approximately 250 mg/kg bw/day (3000 ppm in the feed) for 28 days.

The endocrine disrupting properties demonstrated for many pesticides *in vitro* have only been confirmed *in vivo* for a limited number of pesticides. These include some of the 'old' persistent organochlorine insecticides such as DDT and methoxychlor (Shelby *et al.*, 1996), and some fungicides: procymidon (Gray, Jr. *et al.*, 1999b;Lambright *et al.*, 2000;Ostby *et al.*, 1999), vinclozolin (Gray, Jr. *et al.*, 1999a;Kelce *et al.*, 1994), fenarimol (Andersen *et al.*, 2006;Vinggaard *et al.*, 2005b), and prochloraz (Vinggaard *et al.*, 2002;Vinggaard *et al.*, 2005a). General traits for these pesticides are their ability to react with more than one steroid hormone receptor (Andersen *et al.*, 2002;Gaido *et al.*, 2000;Molina-Molina *et al.*, 2006;Radice *et al.*, 2006;Sohoni and Sumpter, 1998) and to affect the expression of enzymes involved in steroid synthesis and/or metabolism (Delescluse *et al.*, 1998;Sanderson, 2006;Vinggaard *et al.*, 2006;Zachow and Uzumcu, 2006). Whether the ability to act via different mechanisms is a major contributing factor in inducing endocrine disrupting effects *in vivo*, is not known at this time. The importance of the different mechanisms for the integrated *in vivo* response may differ between compounds and may also depend on the developmental stage of the exposed organism.

We have previously demonstrated that another fungicide, fenarimol, had estrogenic properties *in vitro* in the MCF-7 cell proliferation assay as well as *in vivo* where the compound increased uterine weight in ovariectomized female

rats (Andersen *et al.*, 2006). In addition, fenarimol is an anti-androgen *in vitro* (Vinggaard *et al.*, 1999) and causes anti-androgenic effects in the Hersberger assay *in vivo* (Vinggaard *et al.*, 2005b). Fenarimol inhibits aromatase activity (Sanderson *et al.*, 2002; Vinggaard *et al.*, 2000) and affects a range of other cytochrom P450 enzymes including key enzymes involved in steroid hormone biosynthesis (Paolini *et al.*, 1996). Like the azole fungicides, fenarimol was reported to increase progesterone concentration in pregnant rats leading to delayed parturition (WHO, 1995). In some older studies, a reduced fertility of male rats was observed and suggested to be related to an effect in the central nervous system controlling sexual behavior (Hirsch *et al.*, 1987) but the exact mechanism were not explored. No studies on reproductive development in offspring after exposure of pregnant animals to fenarimol have been reported.

The dicarboximide fungicide vinclozolin was one of the first pesticides identified as a potent *in vitro* and *in vivo* androgen antagonist and to disturb sexual development in male pups after perinatal exposure exhibiting reduced AGD, nipple retention and hypospadia (Colbert *et al.*, 2005; Gray, Jr. *et al.*, 1994; Gray, Jr. *et al.*, 1999a; Kelce *et al.*, 1994; Nellemann *et al.*, 2003). Recently, vinclozolin was also reported to virilize female fetuses in mice after prenatal exposure and to up-regulate progesterone receptor mRNA in offspring of both sexes while ER mRNA was down-regulated in females and up-regulated in males (Buckley *et al.*, 2006). Vinclozolin has also been reported to affect the activity of a range of cytochrom P450 enzymes in mice by increasing the activity of some and inhibiting others depending on dose, sex and organ (Hrelia *et al.*, 1996). In addition, vinclozolin was reported to increase the activity of aromatase and mRNA expression in H295R human adrenocortical cells identified (Sanderson *et al.*, 2002). A recent study demonstrated that vinclozolin and its two major metabolites were not only AR antagonists, but also ER agonists and that vinclozolin and one of the metabolites (M2) were progesterone and mineralocorticoid receptor antagonists and M2 was also a glucocorticoid receptor antagonist (Molina-Molina *et al.*, 2006). Hence, also vinclozolin possess the ability to induce endocrine disruption via a range of different mechanisms. Recently, vinclozolin was shown to alter the spermatogenic capacity of male germ cells and sperm viability via its effects on DNA methylation and fetal exposure to vinclozolin during gonadal sex determination in rats caused reduced fertility and sperm development in the adult testis (Uzumcu *et al.*, 2004). Alarmingly, this phenotype was transmitted through the male germ line to at least generation F4 without any further exposure (Anway *et al.*, 2005). Testis from the F2 generation at PND20 had normal morphology but an increase in spermatogenic cell apoptosis. At PND60 (adult) the morphology was still predominantly normal but the germ cell apoptosis was significantly increased (Anway *et al.*, 2006). A similar transgenerational transmission of endocrine disruption was also demonstrated for methoxychlor (another well known endocrine disrupting pesticide) in the same study (Anway *et al.*, 2005) indicating that this phenomenon also might apply to other endocrine disrupters.

In conclusion, all three triazole fungicides included in this study possessed similar properties as prochloraz to act via several endocrine disrupting pathways *in vitro* although the potency in some of the assays were lower than for prochloraz. However, the potency of the triazole fungicides *in vivo* might be higher than predicted from the *in vitro* studies since both tebuconazole and epoxiconazole were capable of inducing effects on reproductive development in the offspring after exposure *in utero*. These effects are likely due to several

different mechanisms operating simultaneously and thereby enhancing the integrated biological response. The lack of effect in the Hershberger assay (where effects on steroid synthesis is omitted by using testosterone supplemented castrated male rats) combined with the effects on reproductive developmental after perinatal exposure strongly indicate that one of the main responsible mechanisms is disturbance of key-enzymes involved in synthesis of steroid hormones. Subtle effects on hormone levels might be without clinical effects in adults because of a tightly controlled homeostasis but could have detrimental effects if they occur under vulnerable stages of reproductive development in the fetus (Sharpe, 2006; Toppari *et al.*, 2006).

Although the endocrine disrupting potency for the individual triazole compounds seems rather low compared to the concentrations of these compounds in the environment and diet, exposure to several azole fungicides (both triazoles and imidazoles) simultaneously is likely due to the wide use of these compounds. Since, the azole fungicides share several mechanisms (e.g., ER and AR antagonism, Ah receptor agonism, and effects on steroid hormone synthesis including inhibition of aromatase) the combined effects induced by these fungicides might be additive. Likewise, they can also add to effects induced by other environmental endocrine disruptors sharing similar mechanisms (Birkhoj *et al.*, 2004; Rajapakse *et al.*, 2002; Silva *et al.*, 2002). Thus, it is important to survey and minimize the exposure of the human population to azole fungicides.

5 Conclusion

The main objective of this study was to investigate endocrine disrupting mechanisms and effects of three frequently used triazole fungicides: tebuconazole, propiconazole and epoxiconazole. The background for the study was our previous findings on the imidazole fungicide, prochloraz, which was demonstrated to possess multiple endocrine disrupting mechanisms, to be anti-androgenic in adult male rats, and to disturb reproductive development in the offspring after perinatal exposure.

Overall the results from this study reveal that also other azole fungicides have the abilities to act as endocrine disruptors via a range of different mechanisms. The affinities of tebuconazole, propiconazole, and epoxiconazole for the ER and AhR were less than for prochloraz while the affinities for the AR were similar. Like prochloraz, all three triazoles caused enhanced production of progesterone and reduced synthesis of testosterone and estradiol *in vitro* in the human adrenocortical carcinoma cell line H295R. Neither prochloraz nor the three triazoles showed convincing interactions with TR. Whether the differences in potencies between prochloraz and the triazole fungicides in some of the *in vitro* assays are due to the triazole ring compared to the imidazole ring and/or related to the number and placement of halogens in the phenyl ring cannot be concluded from the data in this study.

Unlike prochloraz, tebuconazole and propiconazole did not induce anti-androgenic effects *in vivo* in adult male rats in the Hershberger assay. However, tebuconazole and epoxiconazole were capable of inducing effects on reproductive development in the offspring after perinatal exposure. Both compounds caused an increased gestational length and virilization of the female offspring evidenced by increased AGD and the effects resemble those previously observed for prochloraz. Like prochloraz, male offspring was feminized by tebuconazole evidenced by increased retention of nipples and reduced testosterone concentration in fetal testis. The effect of tebuconazole on AGD was less than for prochloraz and at birth AGD was not statistically significant shorter than controls. No feminizing effect on male offspring was seen for epoxiconazole. It appeared to be a potent fetotoxic compound and dams dosed with 50 mg/kg were in general unable to deliver their pups. A growth promoting effect on pups was seen with the lowest dose (increased birth weight, increased AGD) which may be related to the marked up-regulation of testosterone levels in the dams.

Overall the profile of action *in vitro* of the four azole fungicides was relatively similar (except for the AhR response). However, the profile of action *in vivo* in the developmental toxicity studies varied. Tebuconazole possess a more classical endocrine disrupting effect on male pups like the one seen for prochloraz (increased nipple retention, reduced fetal testosterone and increased progesterone). However, epoxiconazole is first of all markedly fetotoxic and secondly it causes altered sex hormone levels in the dams but not in the fetuses. The common features for all three fungicides are the increased gestational length and the virilizing effect on female pups. The lack of effect from the triazoles in the Hershberger assay, the effect on steroid

hormone synthesis in the *in vitro* assay combined with the enhanced maternal plasma concentration of progesterone and the decreased fetal testicular concentration of testosterone after exposure of pregnant rats strongly indicate that interference with key-enzymes involved in steroid hormone synthesis is one of the major mechanism behind the developmental effects in the offspring.

6 Perspectives

6.1 Research perspectives

The results from this study demonstrate that the triazole fungicides, tebuconazole, propiconazole, and epoxiconazole, like the imidazole fungicide prochloraz, are able to act as endocrine disruptors during several pathways. In addition, the results strongly indicate that interference with steroid hormone synthesis is the major mechanism behind the observed effects on reproductive development in offspring after prenatal exposure. The results indicate that inhibition of CYP17 (an enzyme involved in the conversion of cholesterol to progesterone and testosterone) may be one of the targets but other steroidogenic enzymes may also be affected. This issue deserves further studies in order to elucidate which enzymes are affected and the relative impact of this for the total response *in vivo*.

Male fetuses are considered to be more sensitive towards endocrine disruptors than female fetuses because development of the male phenotype requires more hormonal regulation than female development (Sharpe, 2006). However, the two triazoles tebuconazole and epoxiconazole both virilized female offspring while the effects on male offspring were less pronounced. Further studies on virilizing effects on female pups after exposure to endocrine disrupters are wanted to confirm these observations and to elucidate the mechanism behind. The impact of virilization for the female fertility as adults would also be interesting to investigate.

Some phthalates esters (DBP, DEHP, and BBP) disrupt male reproductive development after *in utero* exposure in rats (Foster, 2006) although these substances do not block the AR (Mylchreest *et al.*, 1999). The effects include decreased AGD, nipple retention and malformations of the external genitalia including hypospadias and cryptorchidism. The critical mechanism for induction of these effects is a marked reduction in fetal testicular testosterone production at the critical window for development of the reproductive tract. Hence, in several ways the effects observed for the azole fungicides resembles those seen for the phthalates. Phthalates also had other effect on Leydig cell function including reduced production of insulin-like factor 3 (insl3) and changes in gene-expression of a number of enzymes and transport proteins involved in normal testosterone biosynthesis and transport. It would be obvious to further investigate if azole fungicides have similar effects on Leydig cell function as the phthalates.

The recent observation, that fetal exposure to vinclozolin or methoxychlor during gonadal development caused decreased spermatogenetic capacity, not only in the exposed males, but also in subsequent generations is a matter of concern. Hence, the ability of other endocrine disruptors, inclusive azole fungicides, to reprogram the germ line and to produce a trans-generational effect on reproductive capacity should be investigated. These studies should include examination of sperm production and apoptosis in spermatogenic cells.

6.2 Regulatory perspectives

The endocrine disrupting properties of azole fungicides demonstrated in this study, as well as in our previous studies on prochloraz, identify azole fungicides as a group of endocrine disruptors capable of inducing effects via several different mechanisms. This makes the risk assessment more complex since many different end-points have to be considered but in general we strongly recommend that all azole fungicides are regarded and regulated as endocrine disruptors until the individual substances have been properly tested.

The results from this study indicate that interference with key-enzymes involved in steroid hormone biosynthesis may be a central mechanism behind the observed effects on reproductive development. This illustrates the importance of applying assays for disruption of steroidogenesis when chemicals are screened for potential endocrine disrupting properties, since chemicals with weak or no affinity for the ER and/or AR may still induce endocrine disruption *in vivo* during this mechanism.

Since azole fungicides are commonly used in agriculture, the population might be exposed from residues in food products and from drifting from sprayed areas. In addition, occupational exposure takes place in agriculture and horticulture. The azole fungicides seem to share several modes of actions and therefore the effects might be additive if the population are exposed to more azole fungicides simultaneously. This is very likely to occour via the diet. Hence, there is a need for better surveillance of the exposure situation of these fungicides. This could be obtained by including more of the azole compounds in the surveys of food products. Especially those used in largest amounts, such as epoxiconazol, should be included. In addition, development of biomarkers to investigate the exposure level in occupationally exposed groups and in samples from the general population is warranted. Until more is known about the potential risk of these compounds, efforts should be taken to minimize the exposure of especially women in the childbearing age group as well as children to these compounds.

Prochloraz is no longer approved for sale in Denmark after 2006. However, the compound has not been banned for used by the authorities and therefore remains can be used legally and according to experience from other products this process may take several years. A more effective way to reduce the exposure of the Danish population would be a regular ban, sine this would minimize (ideally eliminate) occupational exposures and exposure from Danish produced food stuffs immediately. In addition, a ban would also give a signal to other countries about the potential health risk assessed for this pesticide.

7 References

Almstrup, K., Fernandez, M. F., Petersen, J. H., Olea, N., Skakkebaek, N. E., and Leffers, H. (2002). Dual effects of phytoestrogens result in u-shaped dose-response curves. Environ.Health Perspect. **110**, 743-748.

Andersen, H. R., Andersson, A.-M., Arnold, S. F., Autrup, H., Barfoed, M., Beresford, N. A., Bjerregaard, P., Christiansen, L. B., Gissel, B., Hummel, R., Jorgensen, E. C. B., Korsgaard, B., Le Guvel, R., Leffers, H., McLachlan, J. A., Moeller, A., Nielsen, J. B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K. L., Perez, P., Skakkebæk, N. E., Sonnenschein, C., Soto, A. M., Sumpter, J. P., Thorpe, S. M., and Grandjean, P. (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ.Health Perspect. **107**, 89-108.

Andersen, H. R., Bonefeld-Jorgensen, E. C., Nielsen, F., Jarfeldt, K., Jayatissa, M. N., and Vinggaard, A. M. (2006). Estrogenic effects in vitro and in vivo of the fungicide fenarimol. Toxicol Lett. **163**, 142-152.

Andersen, H. R., and Nielsen, J. B. (2001). *Udsættelse for bekæmpelsesmidler blandt gartneriansatte kvinder*. Arbejdsmiljørådets Service Center.

Andersen, H. R., Vinggaard, A. M., Rasmussen, T. H., Gjermandsen, I. M., and Bonefeld-Jorgensen, E. C. (2002). Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicology and Applied Pharmocology **179**, 1-12.

Anway, M. D., Cupp, A. S., Uzumcu, M., and Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science **308**, 1466-1469.

Anway, M. D., Memon, M. A., Uzumcu, M., and Skinner, M. K. (2006). Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. J.Androl **27**, 868-879.

Ayub, M., and Levell, M. J. (1987). Inhibition of testicular 17 alpha-hydroxylase and 17,20-lyase but not 3 beta-hydroxysteroid dehydrogenase-isomerase or 17 beta-hydroxysteroid oxidoreductase by ketoconazole and other imidazole drugs. J.Steroid Biochem. **28**, 521-531.

Ayub, M., and Levell, M. J. (1989). Inhibition of human adrenal steroidogenic enzymes in vitro by imidazole drugs including ketoconazole. J.Steroid Biochem. **32**, 515-524.

Birkhoj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, H. R., Dalgaard, M., and Vinggaard, A. M. (2004). The combined antiandrogenic effects of five commonly used pesticides. Toxicology and Applied Pharmocology **201**, 10-20.

Boas, M., Feldt-Rasmussen, U., Skakkebaek, N. E., and Main, K. M. (2006). Environmental chemicals and thyroid function. European Journal of Endocrinology **154**, 599-611.

Buckley, J., Willingham, E., Agras, K., and Baskin, L. S. (2006). Embryonic exposure to the fungicide vinclozolin causes virilization of females and alteration of progesterone receptor expression in vivo: an experimental study in mice. Environ.Health **5**, 4.

Christensen, H. B., Petersen, A., Poulsen, M. E., Grossmann, A., and Holm, M. (2006). *Pesticidrester i fødevarer 2005 - resultater fra den danske pesticidkontrol*.

Colbert, N. K., Pelletier, N. C., Cote, J. M., Concannon, J. B., Jurdak, N. A., Minott, S. B., and Markowski, V. P. (2005). Perinatal exposure to low levels of the environmental antiandrogen vinclozolin alters sex-differentiated social play and sexual behaviors in the rat. Environ.Health Perspect. **113**, 700-707.

Delescluse, C., Ledirac, N., de Sousa, G., Pralavorio, M., Lesca, P., and Rahmani, R. (1998). Cytotoxic effects and induction of cytochromes P450 1A1/2 by insecticides, in hepatic or epidermal cells: binding capability to the Ah receptor. Toxicol Lett. **96-97**, 33-39.

Felig, P., Baxter, J. D., and Frohman, L. A. (1995). *Endocrinology and Metabolism*. McGraw-Hill, New York.

Foster, P. M. (2006). Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int.J.Androl **29**, 140-147.

Gaido, K. W., Maness, S. C., McDonnell, D. P., Dehal, S. S., Kupfer, D., and Safe, S. (2000). Interaction of methoxychlor and related compounds with estrogen receptor alpha and beta, and androgen receptor: structure-activity studies. Mol.Pharmacol. **58**, 852-858.

Gallavan, R. H., Jr., Holson, J. F., Stump, D. G., Knapp, J. F., and Reynolds, V. L. (1999). Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod.Toxicol. **13**, 383-390.

Garrison, P. M., Tullis, K., Aarts, J. M., Brouwer, A., Giesy, J. P., and Denison, M. S. (1996). Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. Fundamental and Applied Toxicology **30**, 194-203.

Ghisari, M., and Bonefeld-Jorgensen, E. C. (2005). Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells. Mol.Cell Endocrinol. **244**, 31-41.

Gray, L. E., Jr., Ostby, J., and Kelce, W. R. (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicology and Applied Pharmocology **129**, 46-52.

Gray, L. E., Jr., Ostby, J., Monosson, E., and Kelce, W. R. (1999a). Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. Toxicol.Ind.Health **15**, 48-64.

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

Gutleb, A. C., Meerts, I. A. T. M., Bergsma, J. H., Schriks, M., and Murk, A. J. (2005). T-Screen as a tool to identify thyroid hormone receptor active compounds. Environmental Toxicology and Pharmacology **19**, 231-238.

Haavisto, A. M., Pettersson, K., Bergendahl, M., Perheentupa, A., Roser, J. F., and Huhtaniemi, I. (1993). A supersensitive immunofluorometric assay for rat luteinizing hormone. Endocrinology **132**, 1687-1691.

Hanger, D. P., Jevons, S., and Shaw, J. T. (1988). Fluconazole and testosterone: in vivo and in vitro studies. Antimicrob.Agents Chemother. **32**, 646-648.

Hirsch, K. S., Weaver, D. E., Black, L. J., Falcone, J. F., and MacLusky, N. J. (1987). Inhibition of central nervous system aromatase activity: a mechanism for fenarimol-induced infertility in the male rat. Toxicology and Applied Pharmocology **91**, 235-245.

Ho, S. M., Leav, I., Ghatak, S., Merk, F., Jagannathan, V. S., and Mallery, K. (1998). Lack of association between enhanced TRPM-2/clusterin expression and increased apoptotic activity in sex-hormone-induced prostatic dysplasia of the Noble rat. Am.J.Pathol. **153**, 131-139.

Hrelia, P., Fimognari, C., Maffei, F., Vigagni, F., Mesirca, R., Pozzetti, L., Paolini, M., and Cantelli Forti, G. (1996). The genetic and non-genetic toxicity of the fungicide Vinclozolin. Mutagenesis **11**, 445-453.

Ishihara, A., Nishiyama, N., Sugiyama, S., and Yamauchi, K. (2003a). The effect of endocrine disrupting chemicals on thyroid hormone binding to Japanese quail transthyretin and thyroid hormone receptor. Gen.Comp Endocrinol. **134**, 36-43.

Ishihara, A., Sawatsubashi, S., and Yamauchi, K. (2003b). Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. Mol.Cell Endocrinol. **199**, 105-117.

Juberg, D. R., Mudra, D. R., Hazelton, G. A., and Parkinson, A. (2006). The effect of fenbuconazole on cell proliferation and enzyme induction in the liver of female CD1 mice. Toxicol Appl.Pharmacol. **214**, 178-187.

Kampmann, J. P., Brøsen, K., Nielsen-Kudsk, F., and Nyborg, N. C. B. (1999). *Basal og Klinisk Farmakologi*. FADL's forlag, København.

Kelce, W. R., Monosson, E., Gamcsik, M. P., Laws, S. C., and Earl Gray, L. (1994). Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicology and Applied Pharmocology **126**, 276-285.

Kim, I. Y., Shin, J. H., Kim, H. S., Lee, S. J., Kang, I. H., Kim, T. S., Moon, H. J., Choi, K. S., Moon, A., and Han, S. Y. (2004). Assessing estrogenic activity of pyrethroid insecticides using in vitro combination assays. J.Reprod.Dev. **50**, 245-255.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., and Kobayashi, K. (2004). Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using chinese hamster ovary cells. Environ.Health Perspect. **112**, 524-531.

Laier, P., Metzdorff, S. B., Borch, J., Hagen, M. L., Hass, U., Christiansen, S., Axelstad, M., Kledal, T., Dalgaard, M., McKinnell, C., Brokken, L. J., and Vinggaard, A. M. (2006). Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. Toxicol Appl.Pharmacol. **213**, 160-171.

Lambright, C., Ostby, J., Bobseine, K., Wilson, V., Hotchkiss, A. K., Mann, P. C., and Gray, L. E., Jr. (2000). Cellular and molecular mechanisms of action of linuron: An antiandrogenic herbicide that produces reproductive malformations in male rats. Toxicol.Sci. **56**, 389-399.

Laws, S. C., Carey, S. A., and Kelce, W. R. (1995). Differential effects of environmental toxicants on steroid receptor binding. Toxicologist **15**, 294.

Leger, J. G., Montpetit, M. L., and Tenniswood, M. P. (1987). Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. Biochem.Biophys.Res.Commun. **147**, 196-203.

Lentza-Rizos, C. (1990). Ethylenethiourea (ETU) in relation to use of ethylenebisdithiocarbamate (EBDC) fungicides. Rev.Environ.Contam Toxicol. **115**, 1-37.

Long, M., Laier, P., Vinggaard, A. M., Andersen, H. R., Lynggaard, J., and Bonefeld-Jorgensen, E. C. (2003). Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line. Toxicology **194**, 77-93.

Marty, M. S., Crissman, J. W., and Carney, E. W. (2001). Evaluation of the male pubertal onset assay to detect testosterone and steroid biosynthesis inhibitors in CD rats. Toxicol Sci. **60**, 285-295.

Mason, J. I., Carr, B. R., and Murry, B. A. (1987). Imidazole antimycotics: selective inhibitors of steroid aromatization and progesterone hydroxylation. Steroids **50**, 179-189.

Miljøstyrelsen (2004). *Bekæmpelsesmiddelstatestik 2003.Salg 2001, 2002 og 2003: Behandlingshyppighed 2003.*

Miljøstyrelsen (2006). *Bekæmpelsesmiddelstatestik 2005.Salg 2003,2004 og 2005: Behandlingshyppighed 2005*. Miljøministeriet.

Molina-Molina, J. M., Hillenweck, A., Jouanin, I., Zalko, D., Cravedi, J. P., Fernandez, M. F., Pillon, A., Nicolas, J. C., Olea, N., and Balaguer, P. (2006). Steroid receptor profiling of vinclozolin and its primary metabolites. Toxicology and Applied Pharmocology **216**, 44-54.

Moser, V. C., Barone S Jr, Smialowicz, R. J., Harris, M. W., Davis, B. J., Overstreet, D., Mauney, M., and Chapin, R. E. (2001). The effects of perinatal tebuconazole exposure on adult neurological, immunological, and reproductive function in rats. Toxicol.Sci. **62**, 339-352.

Mylchreest, E., Sar, M., Cattley, R. C., and Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di(n- butyl) phthalate during late gestation in rats is different from flutamide. Toxicology and Applied Pharmocology **156**, 81-95.

Nellemann, C., Dalgaard, M., Holst, B., Bonefeld-Jorgensen, E. C., and Vinggaard, A. M. (2005). Gene expression changes in rat prostate after activation or blocking of the androgen and estrogen receptor. Mol.Cell Endocrinol. **237**, 25-35.

Nellemann, C., Dalgaard, M., Lam, H. R., and Vinggaard, A. M. (2003). The combined effects of vinclozolin and procymidone do not deviate from expected additivity in vitro and in vivo. Toxicol.Sci. **71**, 251-262.

Nellemann, C., Vinggaard, A. M., Dalgaard, M., Hossaini, A., and Larsen, J. (2001). Quantification of antiandrogen effect determined by Lightcycler technology. Toxicology **163**, 29-38.

Nielsen, E., Thorup, I., Schnipper, A., Hass, U., Meyer, O., Ladefoged, O., Larsen, J. C., and Østergaard, G. (2001). *Children and the unborn child: exposure and susceptibility to chemical substances - an evaluation.* The Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Denmark.

Noriega, N. C., Ostby, J., Lambright, C., Wilson, V. S., and Gray, L. E., Jr. (2005). Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring. Biology of Reproduction **72**, 1324-1335.

O'Connor, J. C., Frame, S. R., and Ladics, G. S. (2002). Evaluation of a 15-day screening assay using intact male rats for identifying steroid biosynthesis inhibitors and thyroid modulators. Toxicol Sci. **69**, 79-91.

Ogawa, K., Asamoto, M., Suzuki, S., Tsujimura, K., and Shirai, T. (2005). Downregulation of apoptosis revealed by laser microdissection and cDNA microarray analysis of related genes in rat liver preneoplastic lesions. Med.Mol.Morphol. **38**, 23-29.

Ostby, J., Kelce, W. R., Lambright, C., Wolf, C. J., Mann, P., and Gray, L. E., Jr. (1999). The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. Toxicol.Ind.Health **15**, 80-93.

Paolini, M., Pozzetti, L., Mesirca, R., Sapone, A., and Cantelli-Forti, G. (1996). Testosterone hydroxylase in evaluating induction and suppression of murine CYP isoenzymes by fenarimol. Archives of Toxicology **70**, 451-456.

Pesticidkontoret, M. (1996a). *propiconazol. Bilag 1.1. Sagens oplysninger og Miljøstyrelsens vurdering. Toksikologi.* Miljøstyrelsen.

Pesticidkontoret, M. (1996b). *Tebuconazol. Bilag 1.1. Sagens oplysninger og Miljøstyrelsens vurdering. Toksikologi.* Miljøstyrelsen.

Pesticidkontoret, M. (1998). *Prochloraz. Bilag 1.1. Sagens oplysninger og Miljøstyrelsens vurdering. Toksikologi.* Miljøstyrelsen.

Pesticidkontoret, M. (2003). *Epoxiconazol. Bilag 1.1. Sagens oplysninger og Miljøstyrelsens vurdering. Toksikologi.*

Pesticidkontoret, M. (2004). *Prochloraz. Sundhedsmæssig vurdering, Bilag 1.b. Sagens oplysninger og Miljøstyrelsens vurdering. Toksikologi.*

Porterfield, S. P. (1994). Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. Environ.Health Perspect. **102 Suppl 2**, 125-130.

Porterfield, S. P. (2000). Thyroidal dysfunction and environmental chemicals--potential impact on brain development. Environ.Health Perspect. **108 Suppl 3**, 433-438.

Promega (2001). CytoTox 96 Non-Radioactive Cytotoxicity Assay. Technical Bulletin No.163, Madison, WI, USA.

Radice, S., Chiesara, E., Frigerio, S., Fumagalli, R., Parolaro, D., Rubino, T., and Marabini, L. (2006). Estrogenic effect of procymidone through activation of MAPK in MCF-7 breast carcinoma cell line. Life Sci. **78**, 2716-2723.

Rajapakse, N., Silva, E., and Kortenkamp, A. (2002). Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ.Health Perspect. **110**, 917-921.

Rifkind, A. B. (2006). CYP1A in TCDD toxicity and in physiology-with particular reference to CYP dependent arachidonic acid metabolism and other endogenous substrates. Drug Metab Rev. **38**, 291-335.

Sadekova, S. I., Tan, L., and Chow, T. Y. (1994). Identification of the aromatase in the breast carcinoma cell lines T47D and MCF-7. Anticancer Res. **14**, 507-511.

Sanderson, J. T. (2006). The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicol.Sci. **94**, 3-21.

Sanderson, J. T., Boerma, J., Lansbergen, G. W., and van den Berg, M. (2002). Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Toxicology and Applied Pharmocology **182**, 44-54.

Schurmeyer, T., and Nieschlag, E. (1984). Effect of ketoconazole and other imidazole fungicides on testosterone biosynthesis. Acta Endocrinol. **105**, 275-280.

Sharpe, R. M. (2006). Pathways of endocrine disruption during male sexual differentiation and masculinization. Best Pract.Res.Clin.Endocrinol.Metab **20**, 91-110.

Shelby, M. D., Newbold, R. R., Tully, D. B., Chae, K., and Davis, V. L. (1996). Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. Environ Health Perspect. **104**, 1296-1300.

Silva, E., Rajapakse, N., and Kortenkamp, A. (2002). Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental Science and Technology **36**, 1751-1756.

Sohoni, P., and Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. J.Endocrinol. **158**, 327-339.

Sonne-Hansen, K., and Lykkesfeldt, A. E. (2005). Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. J.Steroid Biochem Mol.Biol. **93**, 25-34.

Sun, G., Thai, S. F., Tully, D. B., Lambert, G. R., Goetz, A. K., Wolf, D. C., Dix, D. J., and Nesnow, S. (2005). Propiconazole-induced cytochrome P450 gene expression and enzymatic activities in rat and mouse liver. Toxicol Lett. **155**, 277-287.

Toppari, J., Virtanen, H., Skakkebaek, N. E., and Main, K. M. (2006). Environmental effects on hormonal regulation of testicular descent. J.Steroid Biochem.Mol.Biol.

Trosken, E. R., Fischer, K., Volkel, W., and Lutz, W. K. (2006). Inhibition of human CYP19 by azoles used as antifungal agents and aromatase inhibitors, using a new LC-MS/MS method for the analysis of estradiol product formation. Toxicology **219**, 33-40.

Trosken, E. R., Scholz, K., Lutz, R. W., Volkel, W., Zarn, J. A., and Lutz, W. K. (2004). Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. Endocr.Res. **30**, 387-394.

Tully, D. B., Bao, W., Goetz, A. K., Blystone, C. R., Ren, H., Schmid, J. E., Strader, L. F., Wood, C. R., Best, D. S., Narotsky, M. G., Wolf, D. C., Rockett, J. C., and Dix, D. J. (2006). Gene expression profiling in liver and testis of rats to characterize the toxicity of triazole fungicides. Toxicology and Applied Pharmocology **215**, 260-273.

Uzumcu, M., Suzuki, H., and Skinner, M. K. (2004). Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. Reprod.Toxicol **18**, 765-774.

van Casteren, J. I., Schoonen, W. G., and Kloosterboer, H. J. (2000). Development of time-resolved immunofluorometric assays for rat follicle-stimulating hormone and luteinizing hormone and application on sera of cycling rats. Biology of Reproduction **62**, 886-894.

Viard, I., Wehrli, P., Jornot, L., Bullani, R., Vechietti, J. L., Schifferli, J. A., Tschopp, J., and French, L. E. (1999). Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. J.Invest Dermatol. **112**, 290-296.

Vinggaard, A. M., Christiansen, S., Laier, P., Poulsen, M. E., Breinholt, V., Jarfelt, K., Jacobsen, H., Dalgaard, M., Nellemann, C., and Hass, U. (2005a). Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. Toxicol Sci.

Vinggaard, A. M., Hass, U., Dalgaard, M., Andersen, H. R., Bonefeld-Jorgensen, E., Christiansen, S., Laier, P., and Poulsen, M. E. (2006).

Prochloraz: an imidazole fungicide with multiple mechanisms of action. Int.J.Androl **29**, 186-192.

Vinggaard, A. M., Hnida, C., Breinholt, V., and Larsen, J. C. (2000). Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol.Vitr. **14**, 227-234.

Vinggaard, A. M., Jacobsen, H., Metzdorff, S. B., Andersen, H. R., and Nellemann, C. (2005b). Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol. Toxicology **207**, 21-34.

Vinggaard, A. M., Joergensen, E. C., and Larsen, J. C. (1999). Rapid and sensitive reporter gene assays for detection of antiandrogenic and estrogenic effects of environmental chemicals. Toxicology and Applied Pharmocology **155**, 150-160.

Vinggaard, A. M., Nellemann, C., Dalgaard, M., Jorgensen, E. B., and Andersen, H. R. (2002). Antiandrogenic effects in vitro and in vivo of the fungicide prochloraz. Toxicol.Sci. **69**, 344-353.

WHO. Pesticides residues in food - 1995 evaluations. Part II Toxicology and environment. 1995. WHO/JMPR.

Willingham, E., Agras, K., de, S. A., Jr., Konijeti, R., Yucel, S., Rickie, W., Cunha, G. R., and Baskin, L. S. (2006). Steroid receptors and mammalian penile development: an unexpected role for progesterone receptor? J.Urol. **176**, 728-733.

Zachow, R., and Uzumcu, M. (2006). The methoxychlor metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, inhibits steroidogenesis in rat ovarian granulosa cells in vitro. Reprod.Toxicol **22**, 659-665.

Zhang, W., Ramamoorthy, Y., Kilicarslan, T., Nolte, H., Tyndale, R. F., and Sellers, E. M. (2002). Inhibition of cytochromes P450 by antifungal imidazole derivatives. Drug Metab Dispos. **30**, 314-318.