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Environmental Protection Agency

Developmental toxicity effects in experimental animals after mixed exposure to endocrine disrupting pesticides

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Title:

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Content

Preface

The research presented in this report on developmental toxicity effects in experimental animals after mixed exposure to endocrine disrupting pesticides was carried out from April 2008 to May 2010.

The involved institutions were the Division of Toxicology and Risk Assessment and the Division of Food Chemistry at the National Food Institute, DTU Food, and the department of Informatics and Mathematical Modeling, DTU Informatics, at the Technical University of Denmark.

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We would like to thank the members of the steering committee for their involvement throughout the project period. Especially we want to thank the referees: Susanne Hougaard, Karin Sørig Hougaard, Grete Østergaard and Christian Ritz for their constructive comments during the writing process of this report.

Sammenfatning

Baggrund
Der er en stigende bekymring for, om udsættelse for selv lave niveauer af hormonforstyrrende stoffer under fosterudviklingen og i de første levemåneder kan føre til varige skader på reproduktions- og nervesystemet. En række af de stoffer som mistænkes for at være hormonforstyrrende er pesticider og hovedformålet med dette projekt var derfor, i et stort dyreforsøg at undersøge om kombineret udsættelse for hormonforstyrrende pesticider, ved dosisniveauer som for de enkelte stoffer ikke giver nogen effekt, kan føre til blivende hormonforstyrrende effekter i afkommet.

Flere dyreforsøg har vist, at visse pesticider kan forårsage hormonforstyrrende virkninger under udviklingen, men niveauerne af de enkelte pesticider i humane væv synes dog at være til stede ved så lave niveauer, at det indtil videre ikke har givet anledning til bekymring for uønskede reproduktive effekter hos mennesker. Risikovurdering af pesticider er dog i øjeblikket baseret på NOAELs (no observed adverse effect levels) for effekterne af enkeltstoffer, men mennesker udsættes ikke kun for et, men derimod for en blanding af mange hormonforstyrrende stoffer samtidig. Derudover har nyere epidemiologiske undersøgelser vist associationer mellem eksponering for visse organiske pesticider under udviklingen og medfødte misdannelser af kønsorganerne hos drengebørn (Damgaard et al., 2006), hvilket indikerer at kumulativ eksponering for hormonforstyrrende stoffer kan spille en rolle for udviklingen hos mennesker. Disse observationer underbygger resultater fra laboratorieforsøg, hvor der er set alvorlige skader på den reproduktive udvikling efter eksponering for en blanding af hormonforstyrrende kemikalier, selvom hvert af de stoffer som var til stede i blandingen, var det ved lave og ineffektive doser (Silva et al., 2002, Hass et al., 2007, Metzdorff et al., 2007). Disse resultater har væsentlig betydning for risikovurderingen af hormonforstyrrende stoffer, da de peger på, at den nuværende brug af NOAEL for et kemikalie ad gangen kan føre til en undervurdering af den potentielle risiko for mennesker.

I dette projekt blev effekterne af kombineret udsættelse for fem hormonforstyrrende pesticider, alle ved dosisniveauer under NOAEL for effekter på drægtighedslængde, undersøgt i et stort dyreforsøg med drægtige hunrotter. De fem pesticider epoxiconazol, mancozeb, prochloraz, tebuconazol og procymidon er endnu tilladt i EU selvom de alle mistænkes for at være hormonforstyrrende. Udover undersøgelsen af hormonforstyrrende effekter i afkommet indgik der i projektet analyse af, om matematisk modellering af de forventede blandingseffekter kunne give nyttige estimater af effekterne, sammenlignet med de observerede kombinationseffekter. Desuden, blev pesticidindholdet i blodet fra mødre og afkom målt med henblik på at vurdere, om eksponeringen for blandingen forårsagede højere blodniveauer end udsættelse for de enkelte pesticider alene. Projektet omfattede også en undersøgelse af den samme blanding af pesticider i ***in vitro***-analyser, med henblik på at sammenligne resultaterne med dem fra ***in vivo*** forsøget. Endelig blev der, for at vurdere om der er årsag til bekymring i relation til danskernes samlede eksponering for de undersøgte pesticider, udført en sandsynlighedsbaseret undersøgelse af indtaget af de undersøgte pesticider.

Projektet blev udført for at kunne bidrage til regulatoriske overvejelser om behovet for ændringer af procedurer for risikovurdering for pesticider for at tage hensyn til kombinationseffekter og de potentielt alvorlige konsekvenser af kombinationseksponering på udvikling og reproduktion.

Metoder
Drægtige rotter blev doseret fra drægtighedsdag 7 til postnatal dag (PD) 16. Derved blev afkommet eksponeret i foster-tilstanden gennem moderkagen samt i den postnatale periode igennem modermælken. Fire grupper af rotter blev dagligt sonde-doseret, enten med blandingen af de fem pesticider ved forskellige doser (0, 14,58, 29,17 eller 43,75 mg/kg kropsvægt/dag) eller med en høj eller en lav dosis af de enkelte pesticider. Mix-grupperne var på 22 dyr, mens enkeltstofgrupperne indeholdt 10-12 drægtige hunrotter. Den lave dosis af hvert pesticid i enkeltstofgrupperne var den samme dosis som den der indgik i højeste mix-grupppe, mens den høje dosis af enkeltstoffet var 4 gange højere.

Længden af drægtighedsperioden blev registreret, og i den neonatale periode blev afkommet undersøgt for effekter som er følsomme over for anti-androgen påvirkning (anogenitalafstand og bibeholdelse af brystvorter). På ungedag 16, 22 og 50 blev noget af afkommet aflivet og blod og væv blev udtaget til hormon- og kemiske analyser, samt til analyser af organvægte og histopatologi. Endvidere blev misdannelser af kønsorganerne registreret hos hanungerne. Noget af afkommet fortsatte til undersøgelser i adfærdstest. Disse omfattede aktivitetstest, en test af rumlig indlæring og hukommelse, samt en test af parringsadfærd. Også østruscyclus og sædkvalitet samt organvægt og histopatologi af reproduktionsorganerne blev vurderet i det voksne afkom. Kemisk analyse af blodet blev udført ved hjælp af gaskromatografi-massespektrometri, og matematisk modellering af kombinationseffekterne blev udført ved brug af både 'independent action' og 'dose-additivity' modeller.

In vitro blev det i et 'androgen receptor reportergen assay' undersøgt, om pesticiderne aktiverede androgen receptoren (AR) og om de var i stand til at hæmme androgen-induceret aktivering af AR. T-Screen assayet blev brugt til test af skjoldbruskkirtlen receptor aktivitet og pesticiderne blev ligeledes undersøgt for deres effekter på produktion af østradiol, progesteron og testosteron i den menneskelige cellelinie H295R.

Danskernes gennemsnitlige pesticidindtag blev baseret på indtagsberegninger fra den Nationale Kostundersøgelse, samt informationer om restkoncentrationer for de enkelte pesticider pesticid overvågningsprogrammer. Den kumulative eksponering blev beregnet ved hjælp af et computerprogram (Monte Carlo Risk Assessment) og alle indtagsskøn blev justeret i forhold til kropsvægt. 99,9 percentilen blev brugt som referencepunkt og de beregnede eksponeringsværdier blev sammenlignet med DNEL-værdi (derived no effect level) for indexstoffet prochloraz.

Resultater in vivo
Drægtighedslængden var signifikant forøget for de to højeste doser af pestimixen, samt i højdosis epoxiconazol-gruppen, men ikke i nogen andre grupper. Idet den højeste dosis af epoxiconazol var 4 gange højere end den dosis, der indgik i højeste mix-gruppe, viser disse resultater at der var en kombinationseffekt af pesticiderne, på dosisniveauer hvor de enkelte pesticider

ikke medførte nogen effekter.

Bibeholdelsen af brystvorter hos hanafkommet var signifikant højere i alle mix-grupperne, samt ved den højeste dosis af prochloraz, tebuconazol og i begge doser af procymidon, sammenlignet med kontrolgruppen. Da effekten i den højeste mix-gruppe var signifikant højere end effekten af procymidon alene, ved det dosisniveau der indgik i højeste mixdosis, sås også for dette endpoint kombinationseffekter.

Signifikant nedsatte vægte af de hanlige reproduktionsorganer, histopatologiske forandringer samt medfødte misdannelser blev også set i højdosis mix-gruppen, mens disse effekter ikke sås i enkeltstofgrupperne. På PD 16, var vægten af prostata og sædblæren reduceret i den højeste mixgruppe, mens ingen af pesticiderne alene gav statistisk signifikant effekt på disse effektmål. Hos det voksne hanafkom var vægten af reproduktionsorganer også reduceret i højeste mix-gruppe i forhold til kontrolgruppen, mens dette ikke sås i nogen af enkeltstofgrupperne. Derudover var sædcelleantallet reducret i højeste mix-gruppe. Derfor viser resultaterne mix-effekter ved dosisniveauer, hvor de enkelte kemikalier ikke gav effekt.

Øget hyppighed og sværhedsgrad af misdannelser på kønsorganerne blev også fundet ved den højeste mix-dosis. Andelen af hanafkom med misdannelser var markant højere i den højeste mix-gruppe end i nogen af grupperne doseret med de enkelte pesticider alene, i den dosis som indgik i blandingen. Disse resultater viser alvorlig mix- effekt ved et dosisniveau, hvor de enkelte pesticider alene ikke forårsagede tilsvarende effekt.

Modelleringsresultaterne
For effekten 'brystvortebibeholdelse' hos det hanlige afkom, stemte forudsigelsen af mix-effekten baseret på dosis-additivitet overens med de observerede effekter ved lave doser, da der ikke var nogen statistisk signifikante forskelle mellem de forudsagte og de observerede effekter. Men dosis-additivitet undervurderede virkningen for høje doser af blandingen. Independent action forudsigelserne undervurderede i endnu højere grad mix-effekterne ved de høje doser, og overvurderede samtidig effekten ved de lave doser. Et lignende billede blev fundet for modelleringen af drægtighedslængden, hvor dosis-addition forudsigelsen ved lave doser gav en god forudsigelse af de observerede effekter. Både dosis-additivitet og independent action undervurderede mix-effekterne ved høje doser. Men generelt undervurderede dosis-addition forudsigelserne i mindre grad end independent action forudsigelserne.

Kemiske analyseresultater
Procymidon, epoxiconazol, tebuconazol og prochloraz kunne alle påvises i serumprøver fra rottemødrene, og som forventet var serum niveauerne i mødrene generelt højere end i ungerne, idet disse kun blev udsat indirekte, via modermælken. De bedste data var tilgængelige for procymidon og epoxiconazol og de viste, at serum niveauerne i ungerne var omkring 10% af de maternelle niveauer for procymidon, mens de kun var på omkring 0,6% for epoxiconazol.
Ved at sammenligne resultaterne fra mix-gruppeungerne med de unger der blev udsat for enkeltstofferne, sås mix-effekter for procymidon og epoxiconazol. Den samme dosis resulterede nemlig i mere end dobbelt så høje interne doser i ungerne, når pesticiderne blev givet i pestimixen, end når de blev givet alene.

In vitro resultater
Alle pesticiderne bortset fra mancozeb viste AR antagonisme ***in vitro***, med følgende potens: Mix ≈ Procymidon > Prochloraz ≈ epoxiconazol > Tebuconazol. Da indholdet af procymidon i blandingen kun udgjorde ca. 1/3 af den samlede mix-dosis, viser in-vitro resultaterne også kombinationseffekter på AR antagonisme.

I H295 steroidsyntese assayet reducerede både prochloraz, tebuconazol, epoxiconazol og pestimixen testosteron-niveauet i cellerne, mens procymidon og mancozeb ikke havde nogen effekt. Prochloraz, epoxiconazol, tebuconazol og pestimixen reducerede også østradiolniveauerne, mens procymidon og mancozeb ved højere koncentrationer øgede østradiol. Generelt blev alle hormonniveauerne mest påvirket af prochloraz.
I T-Screen sås ingen agonistisk effekt af pestimixen. En svag antagonistisk effekt af pestimixen blev observeret ved koncentrationer på og over 3.13µM, mens koncentrationer over 25µM viste sig at være cytotoksiske.

Kortlægning af pesticidindtag
På trods af usikkerheden i beregningerne af det kumulative indtag af de fire hormonforstyrrende pesticider fra kosten, viste resultaterne at det samlede indtag for danske forbrugere, er under 100% af DNEL-værdien på 25 µgram / kg legemsvægt / dag, for prochloraz for kvinder i den fødedygtige alder mellem 15-50 år.

Konklusioner
Projektet har vist, at kombineret udsættelse under udviklingen for hormonforstyrrende pesticider ved doser under NOAELs for de enkelte pesticider førte til alvorlige effekter på hanungernes kønsudvikling og drægtighedslængden hos mødrene. Undersøgelser af blodprøver fra ungerne tydede på at kombineret udsættelse førte til højere niveauer af pesticiderne i blodet end udsættelse for et pesticid ad gangen. Kombinationseffekterne på drægtighedslængde og brystvorter hos hanungerne kunne prædikteres ved dosis-addition ved de lave doser. ***In vitro*** data viste, at AR reporter gene assay og H295R steroidsyntese assay gav god indikation for de observede ***in vivo*** effekter. Kortlægnigen af pesticid-indtaget i Danmark pegede ikke på grund til bekymring med hensyn til de undersøgte pesticider. Der er dog uvist om den ekstra usikkerhedsfaktor på 3, der blev anvendt for at give plads til menneskers udsættelse for andre hormonforstyrrende stoffer, var tilstrækkelig til at dække de allerede kendte hormonforstyrrende stoffer og især de 100vis til 1000vis af stoffer, som er potentielt hormonforstyrrende baseret på ***in vitro*** data og QSAR modeller. Sammenligninger af ADI for pesticiderne med DNELs for kombinationseffekterne på hanungernes kønsudvikling og drægtighedslængden hos mødrene tyder på at ADI'erne ikke er tilstrækkeligt lave til at beskytte mod kombinationseffekter. Resultaterne fra projektet peger således på at risikovurdering baseret på NOAEL for et stof ad gangen kan undervurdere risikoen og at der er brug for at modificere procedurerne for risikovurdering, så der tages hensyn til kombinationseffekter og de potentielt alvorlige effekter på fosterudvikling og reproduktion hos mennesker.

Summary

Background
There is a growing concern of permanent damage to the endocrine and nervous systems after exposure to even low levels of endocrine disrupting pesticides during pre- and early postnatal development. Risk assessment of industrial chemicals, including pesticides, is currently based on the no observed adverse effect levels (NOAELs) for effects of single compounds. Although animal laboratory experiments have shown that some pesticides can cause endocrine disrupting effects during development, individual pesticides alone may appear to be present in human tissues at too low levels to cause concern for adverse reproductive effects. However, humans are exposed to a mixture of several endocrine disrupting chemicals (EDCs). In addition, there are some indications that cumulative exposure to EDCs may play a role in human development, as epidemiological studies have reported associations between certain organochlorine pesticides and congenital cryptorchidism (Damgaard et al., 2006). These initial observations in epidemiological studies echo findings from laboratory experiments, where substantial mixture effects on reproductive development have been seen even though each of the individual chemicals was present at low, ineffective doses (Silva et al., 2002, Hass et al., 2007, Metzdorff et al., 2007). These findings may have major implications for the human risk assessment of EDCs, as they imply that the current use of NOAELs for single chemicals may lead to an underestimation of the potential risk for humans exposed to mixtures of chemicals.

The main objective of this project was to explore the hypothesis that combined developmental exposure to endocrine disrupting pesticides, at dose levels below NOAELs for the single pesticides, may lead to adverse developmental toxicity effects in rats.

The study was designed to investigate whether a mixture of five environmentally relevant endocrine disrupting pesticides would cause adverse developmental toxicity effects at dose levels below NOAELs for the individual pesticides, and to investigate if mathematic modelling of the expected mixture effects could give useful estimates of the effects, compared to the observed mixture effects. Furthermore, pesticide levels in the blood of dams and offspring were measured in order to evaluate whether the exposure to the mixture caused higher blood levels than exposure to the single pesticides alone. The project also included an investigation of the same mixture of pesticides in ***in vitro*** assays, in order to compare the results with those from the ***in vivo*** mixture study and evaluate the usability of alternative ***in vitro*** methods for estimating potential mixture effects. Finally, in order to evaluate whether there may be a reason for concern in relation to mixed exposure of humans to the investigated pesticides a probabilistic survey of the intake of the studied pesticides and an estimate of the cumulative intake was performed.

All the investigations were performed to give input for regulatory considerations on the need for modification of risk assessment procedures for pesticides in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction.

Methods
The investigated pesticides were epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone. Pregnant rats were dosed from gestation day 7 to postnatal day 16 with either the single pesticides or a mixture of these. Four groups of 22 rats were given daily gavage doses of 0, 14.58, 29.17 or 43.75 mg/kg/day of the mixture, while ten groups of 10 or 12 time-mated rats were similarly dosed with two doses of the individual pesticides. The lowest dose of each pesticide was similar to the dose included in the highest mixture dose and the highest dose of the single pesticides was 4 times higher.

The length of the gestation period was recorded and in the neonatal period the offspring were examined for endpoints sensitive to anti-androgenic action (anogenital distance and nipple retention). At pup day 16, 22 and 50 some of the offspring were sacrificed and blood and tissue was taken for hormone and chemical analysis, organ weights and histopathology. Furthermore a genital malformation score was registered. Some of the offspring continued in the study for behavioural tests, which included activity testing, a test of spatial learning and memory, and a mating behaviour test. Also oestrus cyclicity, semen quality and reproductive organ weights and histopathology were assessed in the adult offspring.
Chemical analysis of the blood was performed using gas chromatography–mass spectrometry, and mathematical modelling of the mixture results was done using both 'independent action' and 'dose addition' models.

In vitro, the ability of the pesticides to activate the androgen receptor (AR) and to inhibit androgen-induced activation of the AR was tested in the androgen receptor reporter gene assay. The T- Screen was used for test of thyroid receptor activity and the pesticides were investigated for effects on the production of estradiol, progesterone, and testosterone in the human adrenocortical carcinoma cell line H295R.

Human intake estimates were based on consumption data obtained from the Danish National Dietary Survey, while residue data for the tested pesticides were obtained from the pesticide monitoring programme, and the cumulative acute exposure was calculated using a Monte Carlo Risk Assessment program. All estimates of possible intakes were adjusted for the individual body weights for each consumer. The 99.9 percentile was used as reference point and the exposures were compared to the DNEL value (derived no effect levels) for the index compound prochloraz.

Results of in vivo study
Gestation length was significantly increased in the two highest mixture groups and in the group exposed to the highest dose of epoxiconazole, but not in any other groups. As the highest dose of epoxiconazole given alone was 4 times higher than the dose included in the highest mixture group, these results showed combination effect of the pesticides at dose levels where the individual pesticides caused no effects.

Nipple retention in male offspring was significantly higher all mixture groups, but also at the highest dose of prochloraz, tebuconazole and both doses of procymidone, compared to controls. Since the magnitude of the effect in the highest mixture group was significantly higher that the effect induced by procymidone alone at the dose level included in the highest mixture, theresults also show mixture effects for this endpoint.

Significant effects on male reproductive organ weight and genital malformations were also seen in the highest mixture group, while no significant effects were seen for the single compound exposure. On PD 16, reduced weights of prostate and seminal vesicle were seen in the highest mixture dose group. None of the pesticides caused statistically significant effects on these endpoints when given alone. In adult male offspring, reduced number of sperm cells and reduced weight of reproductive organs was also seen in the highest mixture dose group compared to controls, but reduced weight of reproductive organs were not seen in any of the groups exposed to the pesticides alone. Therefore, these results show mixture effect at dose levels where the single chemicals did not induce effects.

Increased frequency and severity of genital malformation was also found in the highest mixture group. The percentage of male offspring with genital malformations was markedly higher in the highest mixture group than in any of the groups dosed with the individual pesticides alone at the dose included in the mixture. As such, these results show severe mixture effects at dose levels where the individual pesticides caused no effect when given alone.

Mixture modelling results

For nipple retention in male offspring, the prediction of the mixture effect based on dose-additivity was in agreement with the observed effects at low doses, as there were no statistically significant differences between the predicted and the observed effect doses. However, dose-additivity underestimated the effects for the high doses of the mixture. The independent actions predictions were seen to even stronger underestimate the effects for high doses, and overestimated the effect for the low doses. A similar picture was seen for the endpoint gestation length, where the dose-addition prediction at low doses gave a good prediction of the observed effects. Both dose-additivity and independent action underestimated the mixture effects at high doses. However, the underestimation was generally smaller for the dose-additivity prediction than the independent action prediction.

Chemical analysis results

Procymidone, epoxiconazole, tebuconazole and prochloraz could all be detected in serum samples from dams, and as expected the serum levels in the dams were generally higher than those in the pups, exposed indirectly via maternal milk. The best data were available for procymidone and epoxiconazole and showed based on group means that the serum levels in the pups were around 10% of maternal levels for procymidone, whereas they were only around 0.6% for epoxiconazole.
By comparing the results obtained from the analysis of the pup serum samples from the mixture groups with those from the groups exposed to the pesticides alone, mixture effects were indicated for procymidone and epoxiconazole. The same dose resulted in more than two times higher internal doses in the pups when the pesticides were administrated as part of the mixture than when administrated alone.

In vitro results

All pesticides except for mancozeb exhibited AR antagonism ***in vitro***. The ranking of potencies for AR antagonism was: Mixture ≈ Procymidone > Prochloraz ≈ Epoxiconazole > Tebuconazole. As the content of procymidone in the mixture constituted only approximately 1/3 of the total mixture dose, these results indicated combination effects on AR antagonism ***in vitro***.

In the H295 steroid synthesis assay prochloraz, tebuconazole, epoxiconazole and the mixture was shown to reduce the testosterone level in the cells, whereas procymidone and mancozeb had no effect. Prochloraz, epoxiconazole, tebuconazole and the mixture also reduced estradiol levels, whereas procymidone and mancozeb at higher concentrations caused increased estradiol levels. In general the effects of prochloraz was most pronounced on all the hormones.
In the T-Screen no agonistic effect was observed for the pestimix. A weak antagonistic effect of the pestimix was observed at concentrations at and above 3.13µM. However at concentrations above 25µM cytotoxic effects were reported

Survey of pesticide intake
Despite the uncertainties in the calculations of the dietary cumulative intake of the four endocrine disrupting pesticides, the results indicated that the cumulative intake for Danish consumers is below 100 % of the DNEL of 25 µg/kg bw/day for prochloraz for women in the childbearing age between 15-50 years.

Conclusions
The project has shown that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides caused adverse effects on male sexual development and gestation length in the dams. Investigations of the blood levels of the pesticides in rat offspring indicated that the mixture exposure caused higher blood levels than exposure to the single pesticides alone. The mixture effects on gestation length and nipple retention were well predicted by dose-addition at low doses. The ***in vitro*** data showed that the AR reporter gene assay and the H295R steroidogenesis assay gave good indications for the observed ***in vivo*** effects. The probabilistic survey of the dietary cumulative intake did not show a reason for concern in relation to mixed exposure of Danish consumers to the investigated pesticides. However, it is uncertain, whether the additional uncertainty factor of 3 used to allow room for human exposure to other endocrine disrupting chemicals was sufficient for covering the already known endocrine disrupters and especially the 100s to 1000s of chemicals that are potential endocrine disrupters based on ***in vitro*** data and QSAR modelling. Comparisons of the ADI to the mixture DNELs for effect on nipple retention and gestation length indicate that the ADIs are not sufficiently low to protect against the mixture effects. Thus, the results of the projects implies that risk assessment based on NOAELs for single chemicals can underestimate the risk and that there is a need for modification of risk assessment procedures for pesticides in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction in humans.

Abbreviations and acronyms

AGD	Anogenital distance
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen response element
BW	Body weight
CI	Confidence interval
CHO	Chinese hamster ovary
D	Diestrous
DC-FCS	Dextran-charcoal treated fetal calf serum
DNEL	Derived no effect level
DMSO	Dimethyl sulfoxide
E	Estrous
EDC	Endocrine disrupting chemical
EDEN	Exploring Novel Endpoints, Exposure, Low-dose- and Mixture-Effects in Humans, Aquatic Wildlife and Laboratory Animals (EU project)
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
Epoxi	Epoxiconazol
FCS	Fetal calf serum
GCMs	Gas chromatography–mass spectrometry
GD	Gestational day
GEE	Generalized estimate equations
GL	Gestation length
GLM	Generalized linea model
IC	Index compound
LABC	Levator ani/bulbocavernosus muscles
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOR	Limit of quantification/reporting
M	Metestrous
Mix	Pestimix
MRM	Multiple reaction monitoring
Mz	Mamcozeb
NOAEL	No observed adverse effect level
NOEL	No observed effect level
NR	Nipple retention
OECD	Organisation for Economic Co-operation and Development
P	Proestrous
Peri	Perinatal (from implantation to weaning)
PD	Pup day
PN	Postnatal
PND	Postnatal day
PPloss	Postimplantation loss
Prchl	Prochloraz
Procy	Procymidone
RPF	Relative Potency Factor
RSD_r	Relative repeatability

RSD_R	Relative internal reproducibility
T_3	Triiodothyronine
T_4	Thyroxine
Tebu	Tebuconazol
TG	Test guideline
TH	Thyroid hormone
T-screen	***In vitro*** thyroid assay
UF	Uncertainty factor
US EPA	United States Environmental Protection Agency
VMG NA	Validation and Management Group for Non-Animal Testing

1 Introduction and background

1.1 Objectives and hypothesis

Disruption of hormonal signalling in foetal life can irreversibly affect human development and reproductive health at a later age. Studies have indicated that incidences of disorders in the male reproductive system have been rising within the last 50 years, but the impact of human exposure to endocrine disrupting chemicals (EDCs) is largely unknown at present.

Risk assessment is currently based on the no observed adverse effect levels (NOAELs) for effects of single compounds. Although animal laboratory experiments have shown that some pesticides can cause endocrine disrupting effects during development, individual pesticides alone may appear to be present in human tissues at too low levels to cause concern for adverse reproductive effects. However, humans are exposed to a mixture of several EDCs. In addition, there are some indications that cumulative exposure to EDCs may play a role in human development, as epidemiological studies have reported associations between endocrine disrupting effects such as altered anogenital distance and congenital cryptorchidism, and combined exposure to some phthalates or certain organochlorine pesticides. These initial observations in epidemiological studies echo findings from laboratory experiments with some EDCs where substantial mixture effects on reproductive development occurred even though each of the individual EDCs was present at low, ineffective doses. These findings may have major implications for the human risk assessment of EDCs, as they imply that the current use of NOAELs for single chemicals may lead to an underestimation of the potential risk for humans exposed to mixtures of chemicals.

Presently, there are no data on the effects of combined developmental exposure to endocrine disrupting pesticides having a potential for affecting both reproductive and brain development. Some pesticides acting via an anti-androgenic mechanism mainly seem to disrupt reproductive development, while others acting via disruption of the thyroid hormones may disrupt brain development. However, several pesticides such as some triazoles may act through both mechanisms, while thyroid disrupting pesticides may also have effects on testicular development, and anti-androgenic pesticides may disturb the sexually dimorphic development of the brain. Consequently, it is relevant to study combined effects of pesticides with such dissimilar modes of action.

This project generally aims at exploring the ***hypothesis*** that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides may lead to adverse developmental toxicity effects.

The project has the following main ***objectives***:

1. Investigate whether a mixture of environmentally relevant endocrine disrupting pesticides with dissimilar modes of action causes adverse developmental toxicity effects, including long-term delayed effects, at

dose levels below NOAELs for the individual pesticides in a large extended developmental toxicity rat study.
2. Investigate if modelling of the expected mixture effects based on data for the single chemicals and using dose-addition or independent action give a useful estimate compared to the observed mixture effects for endpoints where this is relevant.
3. Investigate the blood levels of the pesticides in rat dams and offspring to evaluate whether the exposure to the mixture may cause higher blood levels than exposure to the single pesticides alone.
4. Investigate the same mixture of pesticides and the single chemicals using ***in vitro*** assays in order to compare the results with those from the rat mixture study and evaluate the usability of alternative ***in vitro*** methods for estimating potential mixture effects.
5. Provide a probabilistic survey of the intake of the studied pesticides and estimate the cumulative intake to evaluate whether there may be a reason for concern in relation to mixed exposure of humans to the investigated pesticides.
6. Give input for regulatory considerations on the need for modification of risk assessment procedures for pesticides in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction.

1.2 Background

Exposure to EDCs during early life may cause long-term health effects (Murray et al., 2001). The outcome of their action has been shown to have the potential to manifest itself in embryo and foetus and also to have the ability to influence the development of offspring even until it reaches maturity or middle age (Colborn et al., 1993, Cooper and Kavlock, 1997, Murray et al., 2001).

Of considerable concern in Europe is a decline in male semen quality and a high prevalence of congenital malformations of reproductive organs and hormone-dependent cancers (Giwercman et al., 1993, Skakkebaek et al., 2001, Jørgensen et al., 2006). Although animal laboratory experiments have shown that some pesticides can disrupt male sexual differentiation during development, individual pesticides alone have so far not been shown to contribute to adverse human effects at relevant exposure levels. However, some studies indicate increased prevalence of cryptorchidism in sons of women working as gardeners (Weidner et al., 1998) or living on farms where pesticides have been used (Carbone et al., 2007, Kristensen et al., 1997)

Many EDCs have been found as mixtures in humans (Blount et al., 2000, Swan et al., 2005), including children (Brock et al., 2002, Main et al., 2006), and in wildlife (Guillette 2000). Recently, Fernandez et al. (2007) reported an association between cryptorchidism and hypospadia and total estrogenic load measured in mother's placenta extracts. Damgaard et al. (2006) observed an association between congenital cryptorchidism and the levels of certain organochlorine pesticides in mothers' milk. Swan et al. (2005) found that decreases in anogenital distance among male infants are associated with prenatal phthalate exposure. Earlier, Pierik et al. (2004) identified paternal exposures to pesticides and smoking as factors associated with these congenital malformations. These initial observations in epidemiological studies points in the same direction as laboratory experiments with estrogenic or anti-androgenic chemicals where substantial mixture effects occurred even

though each individual chemical was present at low, ineffective doses (Silva et al., 2002, Hass et al., 2007, Metzdorff et al., 2007). Thus, it is important to bear in mind that low exposures to endocrine disrupters may contribute to a combined adverse effect, even though the effects of the single compounds are below the detection limit (Hass et al., 2007, Rajapakse et al., 2002, Silva et al., 2002). These findings have stimulated interest in exploring the consequences of combined exposures to environmentally relevant mixtures of EDCs, including mixtures of pesticides.

Some pesticides such as vinclozolin and procymidone antagonize competitively the AR binding of androgens and affect mainly the reproductive development in male offspring (Simard et al., 1986, Ostby et al., 1999). Other pesticides such as mancozeb and propineb act mainly via disruption of the thyroid hormones and are mainly suspected to disrupt brain development (Hurley et al.,1998, Axelstad et al., 2011). We have recently shown that developmental exposure to PTU, the main metabolite of propineb, induces long-lasting effects on testicular weight (Axelstad et al. in preparation) and on cognitive and hearing functions in the offspring and that these effects correlate with the effects on the thyroid hormone levels during pregnancy (Axelstad et al., 2008).

Our detailed research on prochloraz, combined with studies on other azole fungicides such as tebuconazole and epoxiconazole, indicates that these pesticides have the ability to react through several endocrine disrupting mechanisms, and to induce various endocrine disrupting effects (Vinggaard et al., 2005a, Vinggaard et al., 2005b, Taxvig et al., 2007). We have shown that prochloraz induced anti-androgenic effects in rats ***in vivo*** in a Hershberger test as well as in a developmental toxicity study (Vinggaard et al., 2005a, Vinggaard et al., 2002). Furthermore, the anti-androgenic effect of prochloraz in combination with four other pesticides was found to be additive ***in vivo*** and ***in vitro*** (Birkhøj et al., 2004). Also, binary and tertiary mixtures of anti-androgenic pesticides including prochloraz induced mainly dose-additive effects in the ***in vitro*** androgen-reporter gene assay and in ecotoxicological test systems (Kudsk et al., 2005). Both tebuconazole and epoxiconazole affected reproductive development in the offspring after exposure in utero. Common features for these azole fungicides are that they increase gestational length, virilize female pups, and affect steroid hormone levels in foetuses and/or dams (Taxvig et al., 2007). In addition, our studies indicate that prochloraz may also affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain (Vinggaard et al., 2005a).

Anti-androgenic effects of pesticides on male sexual development may as described above be due to several dissimilar mechanisms of action such as AR-antagonism and effect on steroid hormone levels. Anti-androgenic pesticides may disturb the sexually dimorphic development of the brain and thyroid disrupting pesticides may have effects on testicular and brain development. Consequently, it is considered relevant to study combined effects of pesticides with such dissimilar modes of endocrine action.

2 Research plan and selection of pesticides

2.1 Research plan

The work has proceeded in several steps and an overview of the time schedule is shown in table 2.1.

Table 2.1 Overview of project time schedule

Activities	Endpoints/ activities	2008	2009	2010	2011
Planning of studies	Selection of dose levels etc.	---			
In vitro studies	Anti-androgenic activity: H295 and androgen reporter gene assay; Thyroid assay		---	--	
Initial range-finding studies in rats	General toxicity and pregnancy endpoints	---	---		
Extended developmental toxicity mixture study in rats	Developmental toxicity endpoints General toxicity endpoints in dams		--------	----------	--
Modelling using dose-addition and independent action				---	-
Chemical analysis of blood samples			----	----	
Survey of intake	Probabilistic approach, cumulative intake		--	----	
Data analysis and reporting		---	----------	----------	-----

Initially, small-scale range-finding studies in non-pregnant and pregnant animals were performed in order to minimize potential animal suffering and select relevant dose levels for a the extended developmental study.

Thereafter, the extended developmental toxicity mixture study (in the rest of the report referred to as the extended mixture study) with the pesticides combined at a mixture ratio proportional to the NO(A)ELs for endocrine disrupting effects on gestation lengths and pup survival for the individual pesticides was performed. This combination was studied at doses equal to the NO(A)ELs for endocrine disrupting effects of the individual pesticides and at multiples of this dose. Additionally, the extended mixture study included two

doses of each pesticide in order to compare the effects of the mixture with the effects of the single pesticides within the same study.

In addition, the data for the single pesticides was used together with previous data from our laboratory for modelling the predicted combination effect based on dose-addition and independent action. Chemical analysis of the pesticides (parent compound) in blood samples from dams and offspring were used for evaluating if mixture exposure and exposure to the single pesticides lead to similar blood levels.

In vitro assays of the same mixture of pesticides and the single chemicals were done in order to compare the results with those from the rat mixture study and evaluate the usability of alternative ***in vitro*** methods for estimating mixture effects.

A survey of cumulative intake levels for the pesticides in Denmark was developed and was together with other relevant exposure data and the data from the mixture study used for estimating the risk for adverse effects of combined exposure to the pesticides.

2.2 Selection of pesticides and dose levels

The 5 pesticides in the pesticide-mixture, i.e. procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb were selected based on information on pesticide use in Denmark and EU and data on endocrine disrupting activity and effects (see table 2.2).

Table 2.2 Pesticides included in pesti-mix, use and overview of endocrine disrupting effects based on expertise in the group and published literature

Sale and use in DK (2005)	Endocrine disrupting effects
Epoxiconazole 46-47 tpa	*In vitro*: multiple endocrine disrupting activities *In vivo*: effects on reproductive development in offspring, increased gestational length, virilized female pups, altered steroid hormone levels in foetuses and/or dams, increased perinatal mortality (Taxvig et al., 2007)
Mancozeb 435-481 tpa	*In vivo*: affected thyroid function, decreased T_4 levels in pregnant dams, suspected developmental neurotoxicant (Hurley et al., 1998, Axelstad et al., 2011)
Prochloraz 0.4-1.8 tpa	*In vitro*: multiple endocrine disrupting activities *In vivo*: anti-androgenic effects in a Hershberger test and developmental toxicity study (Vinggaard et al., 2005a, Vinggaard et al., 2002)
Tebuconazole 22-26 tpa	*In vitro*: multiple endocrine disrupting activities *In vivo*: effects on reproductive development in offspring, increased gestational length, virilized female pups, effects on steroid hormone levels in foetuses and/or dams (Taxvig et al.,2007).
Procymidone Not approved in DK. Found as pesticide residue (2001) in Denmark, Greece, Spain, Luxembourg, Austria, Portugal, Sweden	*In vitro*: androgen receptor (AR) antagonist *In vivo*: reduced anogenital distance, increased nipple retention, hypospadias, reduced prostate, testis and epididymal weights, and altered behaviour in male offspring (Ostby et al., 1999)

Before initiating the experimental studies, the mixture ratio and dose levels for the individual pesticides were chosen based on previous knowledge on the dose-response relationship for each of the 5 pesticides. For the range-finding studies the main purpose was to find a combined dose that represented the

pesticides at doses around their individual NOAELs without causing major effects on the pregnant animals or on pup survival. The developmental toxicity data from previous studies in our own laboratory was used and an overview of these data as well the mixture ratio used as starting point for the studies is shown in table 2.3. More detailed dose-response data for these previous studies is shown in table 2.4.

Epoxiconazol, prochloraz and tebuconazol cause effects on pregnancy length and perinatal survival, and the selection of dose levels for these chemicals was based on these endpoints. As the 3 pesticides have similar effects they may in combination be able to induce an effect that can be predicted by dose-addition. However, as this has not been shown before and investigation of this was one of the main purposes of the experimental ***in vivo*** work, the dose levels for these 3 pesticides were chosen around their individual NOAELs. The effects on perinatal mortality at the LOAELs were more marked for epoxiconazol and tebuconazol than for prochloraz. Based on this the doses chosen for epoxiconazol and tebuconazol in the mixture were their NOAELs for these effects, whereas a slightly higher dose of 35 mg/kg/day was chosen for prochloraz.

Neither mancozeb nor procymidone caused effects on pregnancy length or perinatal survival at the doses studied. For mancozeb, a dose level of 50 mg/kg/day in the mixture was initially chosen, because our studies have shown that dose levels of 150 mg/kg/day and higher cause marked acute neurotoxicity in pregnant animals (Axelstad et al., 2011). Due to acute neurotoxicity in the first range-finding study using non-pregnant animals the mancozeb dose was decreased to 25 mg/kg/day in all of the studies in pregnant animals. The dose level for procymidone was chosen mainly based on the anti-androgenic potency in our earlier studies.

Table 2.3 Pesticides included in pestimix, NOAEL and LOAEL for effects on gestation lengths and perinatal mortality seen in earlier studies in our laboratory and the mixture ratio

Pesticide	NOAEL, mg/kg/day	LOAEL, mg/kg/day	Increased gestation lengths, % animals	Perinatal mortality, %	Mixture ratio, mg/kg/day
Epoxiconazole	15	50	60%	90%	15
Mancozeb**	>150	-	0-10%	10-20%	25 (50*)
Prochloraz	25	50	40%	28%	35
Tebuconazole	50	100	50%	55%	50
Procymidone**	>150	-	0-10%	10-20%	50

*Only used in the range-finding study in non-pregnant animals
****** A LOAEL has not been found for these pesticides and consequently the data shown for increased gestation length and perinatal mortality are the range observed for these effects in our previous studies

Table 2.4 Pesticides included in pestimix, dose-response data from previous studies, offspring and litter data (first and second table, respectively)

Offspr. data Group	Dose	Litter size	BirW, M	AGD, M	AGDI, M	BirW, F	AGD, F	AGDI, F	NR, M	NR, F
Study 04-30 *Procy. and prochl.* (N= 14-16 for control and 6-8 for exposed groups)										
1.Control	0	9,5	6,2	20,7	11,2	6,0	10,4	5,7	0,4	12,2
2.Procy-5	5	9,4	6,2	20,2	11,0	5,9	10,3	5,7	0,7	12,1
3.Procy-10	10	11,6	6,2	19,8	10,7	5,9	10,6	5,9	1,4	12,2
4.Procy-25	25	8,7	6,2	18,0	9,8	5,8	10,3	5,7	3,5	12,1
5.Procy-50	50	10,0	6,0	17,1	9,4	5,7	10,1	5,7	7,1	12,3
6.Procy-100	100	9,8	5,9	15,1	8,4	5,7	9,8	5,5	10,1	12,4
7.Procy-150	150	9,4	6,1	13,0	7,1	6,0	10,4	5,7	11,9	12,2
12.Prochl-5	5	9,6	6,2	20,6	11,2	5,7	10,4	5,8	1,5	12,0
13.Prochl-10	10	10,5	6,1	20,5	11,2	5,8	10,5	5,9	1,1	12,2
14.Prochl-25	25	10,7	6,3	21,7	11,8	6,0	11,0	6,1	1,4	12,1
15.Prochl-50	50	9,9	6,4	20,7	11,1	6,2	11,8	6,4	2,7	12,3
16. Proch100	100	10,7	6,2	20,3	11,1	6,0	11,8	6,5	4,3	12,2
St.dev.										
1.Control	0	3,3	0,5	1,0	0,7	0,6	1,0	0,6	0,9	0,2
2.Proey-5	5	3,1	0,4	0,9	0,5	0,3	1,3	0,7	0,9	0,2
3.Procy-10	10	2,1	0,3	1,0	0,5	0,3	1,0	0,5	0,8	0,4
4.Procy-25	25	5,4	0,4	0,9	0,4	0,3	1,0	0,6	2,3	0,1
5.Procy-50	50	3,3	0,8	1,8	0,7	0,7	1,1	0,6	2,0	0,3
6.Procy-100	100	5,1	0,4	1,8	1,0	0,5	0,5	0,4	1,1	0,4
7.Procy-150	150	3,4	0,1	1,6	0,9	0,5	1,1	0,6	1,4	0,4
12.Prochl-5	5	1,5	0,4	1,5	0,8	0,5	0,9	0,4	1,8	0,2
13.Prochl-10	10	3,9	0,4	0,9	0,4	0,3	1,2	0,7	1,2	0,2
14.Prochl-25	25	4,0	0,4	1,2	0,6	0,3	1,3	0,8	2,6	0,2
15.Prochl-50	50	4,6	0,5	1,7	0,8	0,6	1,7	0,8	1,6	0,2
16. Proch100	100	1,2	0,4	0,8	0,3	0,4	0,9	0,5	2,0	0,3
Study 05-16 *Tebu and Epoxi* (N= 6-8 for groups 1-4 and N=1-2 for group 5)										
1: Control	0	11,2	5,6	20,7	11,6	5,5	10,5	5,9	2,1	12,5
2: Tebu-50	50	10,8	5,7	20,6	11,5	5,6	10,9	6,2	3,4	12,5
3: Tebu-100	100	8,8	5,8	21,3	11,9	5,5	11,6	6,6	3,1	12,3
4: Epoxi-15	15	11,0	6,4	22,1	11,9	6,0	11,8	6,5	2,5	12,3
5. Epoxi-50	50	10,0	6,5	20,7	11,1	5,7	10,3	5,8	3,4	12,0
St.dev.										
1: Control	0	1,7	0,3	1,3	0,8	0,3	0,4	0,2	1,6	0,4
2: Tebu-50	50	3,6	0,4	0,7	0,5	0,6	0,7	0,4	0,9	0,4
3: Tebu-100	100	3,8	0,7	0,9	0,9	0,9	0,6	0,4	2,5	0,4
4: Epoxi-15	15	2,2	0,7	1,4	0,9	0,7	0,9	0,5	1,1	0,2
5. Epoxi-50	50	2,8	0,2	1,7	1,0	na	na	na	na	na
Study 07-17 *Mancozeb* (N = 18-22)										
1: Control	0	11,3	6,0	20,8	11,5	5,7	10,7	6,0	0,5	12,4
2: Mz-50	50	10,0	6,1	20,5	11,3	5,8	10,4	5,8	0,7	12,3
3: Mz-100	100	11,5	5,8	20,8	11,6	5,5	10,5	6,0	0,5	12,3
4: Mz150/100	150/100	11,2	5,3	19,3	11,1	5,1	10,3	6,0	0,3	12,4
St.dev.										
1: Control	0	2,6	0,4	1,0	0,5	0,3	0,6	0,4	0,6	0,4
2: Mz-50	50	4,0	0,7	1,3	0,6	0,6	1,0	0,4	1,1	0,3
3: Mz-100	100	3,1	0,3	1,5	0,7	0,4	0,5	0,3	0,5	0,3
4: Mz-50/100	150/100	1,6	0,4	0,7	0,3	0,3	0,3	0,3	0,5	0,2

(BirW= birth weight, F= female pups, M = male pups, AGD = anogenital distance, AGDI= anogenital index, NR = nipple retention, Procy.=procymidone, Prochl.=prochloraz, Tebuc=tebuconazole, Epoxi=Epoxiconanzole, Mz=Mancozeb)

Litter data Group	Dose	GL	Dam bw	%pploss	%-stillborn	%pn dead	%peri dead
Study 04-30 *Procymidone and prochloraz*							
1.Control	0	22,5	249,9	17,2	1,2	2,3	19,3
2.Procy-5	5	22,3	250,8	15,1	3,3	4,4	18,7
3.Procy-10	10	22,5	247,6	6,2	1,3	2,1	8,0
4.Procy-25	25	22,0	249,5	20,3	0,0	0,0	20,3
5.Procy-50	50	22,6	234,3	21,2	0,0	0,9	22,1
6.Procy-100	100	22,6	242,6	13,3	0,0	20,0	23,3
7.Procy-150	150	22,3	233,6	19,5	0,0	2,4	21,3
12.Prochl-5	5	22,4	244,9	14,0	1,3	1,3	14,9
13.Prochl-10	10	22,5	247,1	13,3	0,0	1,4	14,6
14.Prochl-25	25	22,7	238,6	28,6	0,0	4,2	32,1
15.Prochl-50	50	23,4	238,4	19,6	8,1	15,2	27,6
16. Prochl-100	100	23,3	228,8	2,9	22,0	25,4	27,9
St.dev.							
1.Control	0	0,6	19,8	14,1	4,7	7,2	13,9
2.Proey-5	5	0,5	19,8	12,2	6,2	6,3	14,2
3.Procy-10	10	0,5	17,2	9,5	3,5	4,0	11,2
4.Procy-25	25	0,0	15,7	23,4	0,0	0,0	23,4
5.Procy-50	50	0,7	18,5	19,6	0,0	2,5	18,6
6.Procy-100	100	0,5	9,2	20,9	0,0	44,7	43,1
7.Procy-150	150	0,5	28,0	34,1	0,0	4,1	33,9
12.Prochl-5	5	0,5	21,5	8,1	3,4	3,4	10,3
13.Prochl-10	10	0,5	17,1	10,6	0,0	3,9	10,7
14.Prochl-25	25	0,5	13,7	35,4	0,0	8,1	33,1
15.Prochl-50	50	0,5	29,0	18,8	18,6	37,5	34,4
16. Prochl-100	100	0,5	19,6	4,6	39,2	38,8	37,9
Study 05-16 *Tebuconazol and epoxiconazol*							
1: Control	0	22,5	242,8	6,6	2,0	3,4	9,7
2: T-50	50	22,7	230,3	10,3	0,9	3,4	13,4
3: T-100	100	23,4	222,3	27,3	3,8	27,0	55,0
4: E-15	15	22,7	229,2	16,0	0,9	2,8	18,2
5. E-50	50	23,7	222,0	34,2	61,1	69,4	88,8
St.dev.							
1: Control	0	0,5	23,5	5,1	4,0	5,5	8,0
2: T-50	50	0,5	19,5	11,2	3,2	7,0	12,5
3: T-100	100	1,2	16,0	23,5	8,0	37,5	36,9
4: E-15	15	0,7	21,7	30,0	2,8	5,9	30,0
5. E-50	50	0,8	18,4	18,2	47,4	52,9	29,7
Study 07-17 *Mancozeb*							
1: Control	0	22,6	238,0	8,8	0,0	2,1	10,7
2: Mz-50	50	22,9	233,1	17,4	5,0	7,4	21,3
3: Mz-100	100	22,8	221,3	10,8	6,0	6,2	16,5
4: Mz-50/100	150/100	22,7	198,3	14,8	0,0	0,0	14,8
St.dev.							
1: Control	0	0,5	16,2	8,4	0,0	6,0	10,1
2: Mz-50	50	0,4	21,4	21,3	22,4	23,2	26,5
3: Mz-100	100	0,5	20,4	21,5	21,1	21,6	28,1
4: Mz150/100	150/100	0,5	23,0	30,6	0,0	0,0	30,6

(GL = gestation length, bw = body weight, pploss = postimplantation loss, pn = postnatal, peri = from implantation to weaning. Data show % affected litters)

3 *In vivo* studies

3.1 Introduction

The extended developmental toxicity model in the rat has previously been employed by our group to characterize the effects of developmental exposure to EDCs on male offspring in the EU project EDEN. Detailed mixture experiments revealed that statistically significant mixture effects were observed when anti-androgens were combined at levels below their individual no-observed-effect-levels (Hass et al., 2007, Metzdorff et al., 2007).

Most of the endpoints studied are similar to those included in OECD guidelines for reproductive and developmental toxicity (OECD TG 416 and OECD TG 426), while others such as nipple retention in male pups, mating behaviour and hormone levels have been included due to their sensitivity to endocrine disrupters. An overview of the studies and the endpoints is given in table 3.1.

Table 3.1 Overview of the studies

Study design	Endpoints studied in adults/dams	Endpoints studied in offspring, pup day (PD) 1-22	Endpoints studied in offspring after PD 22
Range-finding, non-pregnant animals, control and 3 doses of the mixture, N = 6	Body weight gain, clinical observations	Not included	Not included
Range-findings, two studies in pregnant animals, control and 5 doses of the mixture, N = 8 time-mated females	Body weight gain during pregnancy and lactation, gestation length, litter size	Birth weight, anogenital distance (AGD), nipple retention (NR), growth and survival	Not included
Extended mixture study, pregnant animals, control, 3 doses pesti-mix, N= 22 time- mated females; 2 doses of the 5 pesticides, N = 10 or 12 time-mated females	Body weight gain during pregnancy and lactation, gestation length, litter size,	Birth weight, AGD, NR, growth and survival, malformations (hypospadia), weight and histopathology of reproductive organs and thyroid, chemicals and hormones in blood	Sexual maturation, oestrus cyclicity, motor activity, learning and memory, mating behaviour, semen quality, weight and histopathology of reproductive organs

The assessment of body weight gain, pup survival and the clinical observations (e.g. lacrimation, ruffled fur, abnormal gait) were included for assessing the general wellbeing of both the dams and the offspring. Litter sizes and pup birth weights provided data on general reproductive or developmental toxicity effects. The gestation length is a hormone sensitive endpoint in the dams and has earlier been shown by us and others to be affected by pesticides such as prochloraz. In addition, this effect seems to be related to changes in levels of progesterone in the dams. Anogenital distance, nipple retention, weight and histopathology of reproductive organs,

malformations such as hypospadias, and semen quality are sensitive endpoints for anti-androgenic effects. Analysis of hormone levels of testosterone, estradiol and progesterone in blood samples was included for assessing disruption of the hormonal homeostasis and for comparison with the ***in vitro*** studies. Chemical analysis of the blood was included to assess internal doses of the pesticides. The assessment of the behavioural endpoints was included for revealing potential effects induced by the anti-androgenic activity on the sexual dimorphic development of the brain. In addition, these endpoints are together with pup body weight the most sensitive endpoints for the effects of thyroidal disruption during development. Weight and histopathology of the thyroid and thyroid hormone (TH) levels provide a direct measure of thyroidal disruption.

The endpoints assessed cover effects on male and female offspring during the postnatal development of the pups as well as long-lasting effects in the adult offspring. Generally, any toxicity to the dams was assessed and evaluated in relation to the developmental toxicity effects.

3.2 Materials and methods

3.2.1 Range-finding study in non-pregnant female animals

Four groups of 6 young female Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were given daily gavage doses of 0, 150, 200 and 250 mg/kg/day of the mixture of the 5 pesticides for 22 days. The mixture ratio was based on the estimated NOAELs for effects on dams and parturition for the individual pesticides. Group 3 was given this mixture, whereas group 2 and 4 received 75% and 125%, respectively (table 3.2).

Due to clear neurotoxic effects after 4 days dosing with 200 mg/kg and especially 250 mg/kg (see results section 3.3.1), the dosing was lowered after day 4 to 150 mg/kg for all animals giver the mixture of pesticides. The animals were observed twice daily for signs of toxicity and body weights were recorded daily.

Table 3.2 Composition of pesticide mixture and dose levels used for range-finding in non-pregnant female animals (doses are mg/kg bw/day)

Pesticide	Group 2	Group 3*	Group 4*
Epoxiconazol	11,25	15	18,75
Mancozeb	37,50	50	62,50
Prochloraz	26,25	35	43,75
Tebuconazol	37,5	50	62,5
Procymidone	37,5	50	62,5
Pestide mixture	150	200	250
% of NOAEL for effect on parturition	75%	100%	125%

* Changed to group 2 dosing from day 5 due to acute neurotoxicity

3.2.2 Range-finding study 1 in pregnant animals

Four groups of 8 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark) were given daily gavage doses of 0, 131.25, 175 and 218.75 mg/kg/day of the mixture of the 5 pesticides from gestation day (GD) 7. The mixture ratio was based on the estimated NOAELs for effects on dams and parturition for the individual pesticides. However, the

amount of mancozeb was decreased to half of the dose used in the range-finding study in non-pregnant females. This was done in order to avoid neurotoxic effects in the dams, since we (based on our previous studies) suspected Mancozeb to be the compound responsible for the acute neurotoxic effects. Thus the mancozeb dose in the highest mixture dose in this study was lower than the lowest dose of mancozeb in the range-finding study in non-pregnant animals (31.25 mg/kg/day compared to 37.5 mg/kg/day). Group 3 was given this mixture, whereas group 2 and 4 received 75% and 125% of this mixture, respectively (see table 3.3)

Due to clear neurotoxic effects after 4 days dosing (GD 10) in the group dosed with 218.25 mg/kg (see resultssection 3.3.2), the dosing of this group was lowered to 175 mg/kg/day from GD 11. The animals were observed twice daily for signs of toxicity and body weights were recorded daily.
For the animals giving birth, the endpoints included time of birth, body weight of the dam, number of implantation scars in the uterus of the dam, number of dead and live born offspring, sex ratio in the litters, body weights of pups, and anogenital distances in the newborn pups.

Due to marked problems with giving birth in most of the animals dosed with these doses of mixture of the pesticides (see results section 3.3.2), caesarean sections were for animal welfare reasons performed for these animals. The endpoints recorded included number of implantations, number of dead and live foetuses, sex ration in the litters, and foetal body weights for the live foetuses.

Table 3.3 Composition of pesticide mixture used for 1st range-finding in pregnant female animals (doses are mg/kg/day)

Pesticide	Group 2	Group 3	Group 4*
Epoxiconazol	11,25	15	18,75
Mancozeb	18,75	25	31,25
Prochloraz	26,25	35	43,75
Tebuconazol	37,5	50	62,5
Procymidone	37,5	50	62,5
Pesticide mixture	131,25	175	218,75
% of NOAEL for effect on parturition	75%	100%	125%

* Changed to group 3 dosing from day 5 (GD 11) due to acute neurotoxicity in the dams

3.2.3 Range-finding study 2 in pregnant animals

Three groups of 8 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark) were given daily gavage doses of 0, 43.75 and 87.50 mg/kg/day of the mixture of the 5 pesticides from GD 7 meaning that they received 25% and 50% of the mixture, respectively (see table 3.4). The animals were observed twice daily for signs of toxicity and body weights were recorded daily.
For the animals giving birth, the endpoints included time of birth, body weight of the dam, number of implantation scars in the uterus of the dam, number of dead and live born offspring, sex ratio in the litters, body weights of pups, anogenital distances in the newborn pups, nipple retention on pup day (PD) 13 as well as reproductive organ weights and score of genital dysgenesis after section on PD 13.

Table 3.4 Composition of pesticide mixture used for 2nd range-finding in pregnant female animals (doses are mg/kg bw/day)

Pesticide	Group 2	Group 3
Epoxiconazol	3,75	7,5
Mancozeb	6,25	12,5
Prochloraz	8,75	17,5
Tebuconazol	12,5	25
Procymidone	12,5	25
Pestide mixture	43,75	87,5
% of NOAEL for effect on parturition	25%	50%

3.2.4 Extended mixture study, low doses

The study included 14 groups and was performed in 4 blocks with a week between each block and the 14 groups were as equally as possible distributed among the 4 blocks. The animals used were 198 time-mated nulliparous, young adult animals (HanTac:WH, Taconic Europe, Ejby, Denmark).

In figure 3.1 an overview of the extended mixture study is given. Four groups of 22 rats were given daily gavage doses of 0, 14.58, 29.17 and 43.75 mg/kg/day of the mixture of the 5 pesticides from gestation day (GD) 7 to postnatal day (PND) 16 (table 3.5). Ten groups of 10 or 12 time-mated rats were similarly dosed with two doses of the individual pesticides (table 3.6). The lowest dose of each pesticide was similar to the dose included in the highest mixture dose and the highest dose of the single pesticides was 4 times higher.

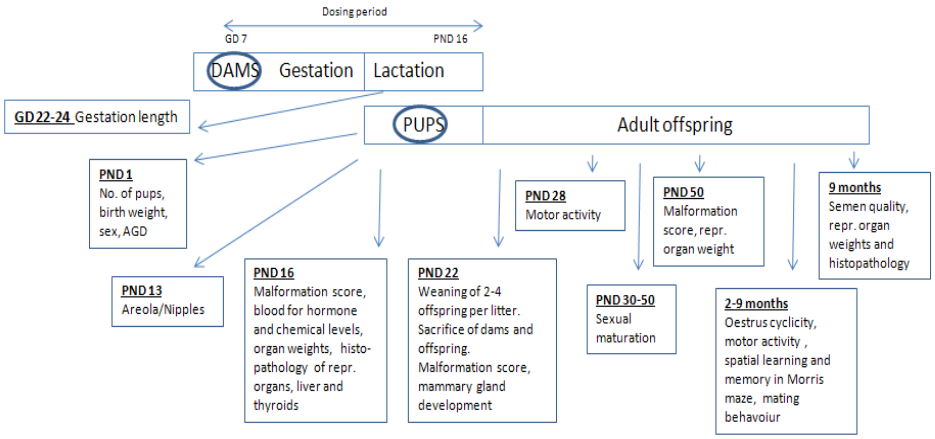


Figure 3.1. Schematic overview of the design of the extended mixture study. Pregnant dams were dosed from gestation day 7 to postnatal day 16. Offspring were examined for AGD, nipples and postnatal growth. At pup day 16, 22 and 50 some of the offspring were sacrificed and blood and tissues were taken for hormone and chemical analysis, organ weights and pathology. Furthermore a malformation score was registered. Some of the offspring continued in the study for behaviour tests, oestrus cyclicity and semen quality tests.

Table 3.5 Composition of pesticide mixture used for the extended mixture study in pregnant female animals (doses are mg/kg/day)

Pesticide	Group 2	Group 3	Group 4
Epoxiconazole	1,25	2,50	3,75
Mancozeb	2,08	4,17	6,25
Prochloraz	2,92	5,83	8,75
Tebuconazole	4,17	8,33	12,50
Procymidone	4,17	8,33	12,50
Pesticide mixture	14,58	29,17	43,75
% of NOAEL for effect on parturition	8,3%	17%	25%

Table 3.6 Group and doses of individual pesticides used in the extended mixture study in pregnant female animals (doses are mg/kg/day)

Group	Pesticide	Dose
5	Epoxiconazole	3,75
6	Epoxiconazole	15
7	Mancozeb	6,25
8	Mancozeb	25
9	Prochloraz	8,75
10	Prochloraz	35
11	Tebuconazole	12,5
12	Tebuconazole	50
13	Procymidone	12,5
14	Procymidone	50

3.2.5 Materials and methods, all studies (when relevant)

3.2.5.1 Animals and chemicals

The animals were housed in pairs until GD 18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 x 27 x 43cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lux, temperature 21 ± 2°C, humidity 50% ± 5%, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) was provided ***ad libitum***. The animals were observed twice daily for signs of toxicity and body weights were recorded daily.

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

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

On the day after arrival (GD 4), the time-mated animals were pseudorandomly distributed into groups with similar body weight (bw) distributions. The weights of dams and individual pups were recorded after delivery and the pups were counted, sexed, and checked for anomalies. Pups

found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD 23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as most of the animals gave birth on GD 23.

Body weight of pups was recorded on days 6, 13, 22 and after weaning. At the age of 22 days 2 males and 2 females from each litter were when possible selected randomly for weaning. These pups were housed in pairs of the same sex and exposure status. Dams and the rest of the pups were sacrificed on PD 22.

3.2.5.2 Anogenital distance and nipple retention

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

3.2.5.3 Estrous cyclicity

Vaginal smears were collected every day between 8 and 10 am for 15 consecutive days starting when the female offspring were 22-23 weeks old. A swab moistened in saline was inserted in to the vaginal lumen and cells were transferred to a glass microscope slide. The samples were then allowed to air dry. When dry, the smears were fixed in 96% ethanol and stained with Gill's hematoxylin, Orange G6 and eosin-azure 50 (provided by VWR, Gentofte, Denmark) according to the adapted Papanicolaou (PAP stain) procedure reported by (Hubscher et al., 2005). After staining the smears were mounted in Eukit. The stained smears were examined by light microscopy and stages were classified as Estrous (E), Metestrous (M), Diestrous (D) or Proestrous (P) or transitions between stages. These stages were recognized by the presence, absence or proportional numbers of epithelial cells, cornified cells and leucocytes as described in OECD guidance document 106.

3.2.5.4 Behavioural Testing

The investigations were performed during the animals' dark cycle, i.e. their active period, and the experimenter was kept unaware as to which group an individual rat belonged. Exposed and control animals were tested alternately and so was female and male animals. The males tested for learning and memory and mating behaviour was the same. The number of offspring in these behavioural tests was: N=10 in control and mixture groups and N=2-6 in the groups dosed with the single pesticides.

Motor activity and habituation capability. 1-2 males and females from each litter were tested on PD 28 and at the age of around 2 months. The activity of the animals was recorded in activity boxes with photocells for 10 x 3 min. The total activity during the 30 min was used as a measure of general activity. In order to assess habituation capability, the 30 min was divided into two time periods of 15 min.

Learning and memory (Morris water maze). The animals were tested at age of 4-5 months in a maze with a diameter of 220 cm as described earlier (Hass et al., 1995, 1999) with minor modifications. The pool was filled with water at room temperature (20°C). Four points on the rim of the pools, N, E, S and W (not true magnetic directions), were used as starting points and divided the pool into four arbitrary quadrants. A circular transparent platform was

situated on a solid support and submerged 1 cm below the water surface, and thus invisible from water level. The animals were tested in four daily trials using the four starting points assigned in a pseudo-random sequence. When the rat swam to and climbed onto the platform, the trial was completed. If the animal failed to locate the platform within 60 s, it was led to the platform. All animals were left to sit on the platform for 15 s, before it was returned to the cage. A video-tracking device (Viewpoint video tracking system, Sandown Scientific, Middlesex, England) tracked the route of the animals, and the latencies to find the platform, the path lengths and swimming speeds were used as end points.
The following scheme was used:
Learning. With the platform situated at the centre of SW quadrant, the animals were trained until a stable performance was established, i.e. 4 trials per day for 7 days (five consecutive days, two day break and then two more days of training).
New platform position (reversal learning): The day after the last memory test, the animals were tested in a reversal procedure with the platform placed opposite the original location, i.e. in the centre of the NE quadrant. The animals were tested for 4 trials a day for two consecutive days.

Mating behaviour in male offspring. The male rats needed to be sexually experienced before the mating session as described by Chahoud and Faqi (Chahoud and Faqi 1998). For the assessment of male mating behaviour, non-ovariectomized sexually mature female Wistar rats (weight 170 g ± 20g) were purchased from Taconic Europe. These female rats were treated with β-oestradiol-3-benzoate (25 μg/rat) 48 hours before the mating and progesterone (500 μg/rat) 4 hours before mating to become in 'chemical oestrus'. Both solutions were injected subcutaneously. The effects of progesterone dosing last for about 4 hours and the females could therefore be used several times per day. The males were placed with a female in 'chemical oestrus' for 20 minutes. It was required that the female showed proceptive behaviour (described below) otherwise it was replaced by another female rat. The male rats had one training trial in the test described below and the second trial was the mating behaviour session scored as described below.
The mating test was performed between 12 and 16 am (in the active period of the animals) and the female rats were brought in "chemical oestrus" in relation to that time point. The male from the couple was placed in a transparent polycarbonat cage (59.5 x 38 x 20cm (D x W x H)) with a flat lid and with no bedding in an unlit room. A female in "chemical oestrus" was then introduced into the male cage. The same cage was used for all animals in the study. The mating behaviour was recorded using a standard Phillips CCD-MOS video camera (black and white) with sensitivity in the infra- red area (800-950 nm). The camera was placed so that it could record all behaviour from the side of the cage. It was connected to a hard disk/DVD recorder (LVW-545 HDD+DVD recorder) and the recordings of mating behaviour were saved at this recorder. After 20 minutes the female was removed.

All mating behaviour data were scored using a Psion Workabout (ProInfo) with the software Pocket Observer® (Noldus, The Netherlands) installed. This was done by trained observers blind to experimental groups until data processing in The Observer program was completed. The observer looked at the recordings of mating behaviour on a computer and registered the behavioural elements by pressing the following buttons on the Psion Workabout: M= Mount; I= Intromission and E= Ejaculation.

3.2.5.5 Section on PD 13 in range-finding study 2, organ weights and assessment of genital dysgenesis of male external genitalia

The offspring were weighed and decapitated after CO_2/O_2 anaesthesia. Testis, epididymis, ventral prostate, seminal vesicles, and liver were excised and weighed from one male per litter. From one female per litter the uterus and ovary were excised and weighed. The external genitalia of all male offspring were inspected for genital dysgenesis and scored on a scale from 0 to 3, with the observer being blinded with respect to dose group. The scores were:
Score 0 (no effect): Normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact.
Score 1 (mild dysgenesis of the external genitals): A small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening was observed, estimated 0.5-1.4 on an arbitrary scale. The size of the genital tubercle was decreased.
Score 2 (moderate dysgenesis of the external genitals): The preputial cleft was larger, estimated 1.5-2.4 on an arbitrary scale. The urethral opening was situated half-way down towards the base of the genital tubercle (hypospadias).
Score 3 (severe dysgenesis of the external genitals): The preputial cleft was large, estimated 2.5-3.5 on an arbitrary scale. The urethral opening was situated further than half-way down the inferior side of the genital tubercle to the base of the genital tubercle (hypospadia). At the base of the genital tubercle a groove extending laterally was observed (similar to control females at PD 13).

3.2.5.6 Section on PD 16 in extended mixture study

On PD 16, 1-3 male and 1-3 female pups per litter were randomly selected for autopsy. Pups were weighed, decapitated and blood was collected for hormone measurement and analysis of pesticide levels. Male reproductive organs were examined macroscopically for anomalies and testicular descent and scored for the degree of penile malformations on a scale from 0 to 3 with the observer being blinded with respect to dose group (scores as described for PD13). Gubernacular length was determined as a measure of testicular descent, as an increased gubernacular length would indicate chryptorchidism.

Uterus, ovaries, thyroids and liver were dissected from one female pup per litter. Uterus, ovaries and livers were weighed, whereas the thyroid was excised on the thyroid cartilage in order to obtain optimal histological preservation. Uterus, one ovary, alternately left and right, a section of the liver and the thyroid were fixed in formalin and processed for paraffin embedding. Testes, epididymides, ventral prostate, seminal vesicle, levator ani/ bulbocavernosus muscle (LABC), bulbourethral glands, liver and thyroids were dissected from one male pup per litter and weighed. Testes were fixed in Bouin's fixative or formalin and processed for paraffin embedding. Alternately right and left testes were placed in each fixative. Epidiymides, seminal vesicles and thyroids (cleared from the thyroid cartilage) were fixed in formalin and processed for paraffin embedding.

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

A semi-quantitative assessment of seminiferous tubule formation in PD 16 male animals was carried out including all mixture groups as well as single compound groups. This was carried out by measuring tubular diameter and evaluating lumen formation (percentage of tubule cross-sections with lumen)

in 100-120 randomly selected seminiferous tubules per testis. Four randomly selected areas of each testis were photographed at 10x magnification and Image Pro Plus 7.0 software was used for image analysis. Measures were done by an observer blinded to treatment groups.

3.2.5.7 Sections on PD 22 and 50

On PD 22, dams were decapitated in CO_2/O_2 anesthesia and the numbers of uterine implantations were counted. The male and female pups not to be kept after weaning were decapitated on PD 22 in CO_2/O_2 anesthesia and blood samples were collected for hormone analyses. Male reproductive organs were examined macroscopically for anomalies and testicular descent and scored for the degree of penile malformations on a scale from 0 to 3. Gubernacular length was determined as a measure of testicular descent, as an increased gubernacular length would indicate chryptorchidism.

On PD 22 and 50, up to one male and one female per litter from group 1 to 4 were used for investigation of effects on mammary gland development. The 4th abdominal mammary gland was excised for whole mount preparation and histological analysis, alternately from the left and right for each purpose. The mammary analyses are a part of another project and will be reported elsewhere. Also males sectioned on PD 50 were examined macroscopically for anomalies and testicular descent and scored for the degree of penile malformations on a scale from 0 to 3.

3.2.5.8 Section of adult female offspring

Female offspring were autopsied when they were approximately 6 months of age. Stage of estrous cycle was determined using impedance measurement. When an impedance value was higher than 3, the female was assumed to be in proestrus and was sacrificed. Animals were decapitated in CO_2/O_2 anesthesia. Trunk blood was collected in Na-heparine coated tubes and centrifugated for 10 min, 4000 rpm at 4^{o}C. Plasma samples were stored at -20^{o}C for later hormone analysis if relevant. It was noted if no uterine dilatation was seen since it may indicate that the animal may not be in proestrus despite the high impedance value measured. Uterus, liver and alternately the right or left ovary were cleared of surrounding tissue and weighed. One ovary, thyroid (on the thyroid cartilage) and specimens of uterus and liver were fixed in formalin and later embedded in paraffin. The remains of uterus and liver were placed in tubes and frozen in liquid nitrogen for epigenetic analysis as a part of another project that will be reported elsewhere.
Histopathological evaluation was made of the thyroid. One section of the thyroid from rats belonging to group 1, 4, 7 and 8 was stained with hematoxylin and eosin for histological evaluation by an examiner blinded to treatment groups.

3.2.5.9 Section of adult male offspring

Sexually mature male offspring (261-280 days of age ~ around 9 months of age) were weighed, anesthetized in CO_2/O_2 and decapitated. Immediately after decapitation, trunk blood was collected in Na-heparine coated tubes. After centrifugation for 10 min, 4000 rpm at 4^{o}C, plasma samples were stored at -20^{o}C for later hormone analysis if relevant. The external genitals were inspected for anomalias: descendent testes, alopecia in the perineal area, cleft phallus, and hypospadia. Malformations were scored using a system with 0 denoting the normal and 3 the most severe changes. The observer was blinded with respect to dose groups. Semen motility was analyzed in all dosed groups, whereas the endpoint sperm counts was only performed in the control males

and in the males from group four, the highest mix dose. For sperm motility analysis, spermatozoa were obtained from the distal cauda and sperm samples were prepared and analysed by computer assisted sperm analysis (CASA) as described in Jarfelt ***et al.*** (2005). For sperm count analysis, cauda epididymis was weighed and prepared as described by Jarfelt ***et al.*** (2005), and samples were analysed using 10× UV fluorescent objective and IDENT OPTIONS. Ten fields were analysed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda.
The rats were further authopsied and macroscopically examined. The following organs were excised and weighed: right and left testis, ventral prostrate, seminal vesicles with seminal fluid, epididymis, bulbourethral glands, levator ani/bulbocavernosus muscles (LABC), liver and thyroid. Samples of visceral (omental) and abdominal subcutaneous adipose tissue collected between third and fourth mammary glands were weighed and transferred to test tubes containing RNAlater®. The brain was excised and stored for a possible later investigation of kisspeptin as part of a PhD project. Alternately, the right or left fourth abdominal mammary gland (exclusive lymph node) was excised, weighed and placed in RNA later®. The contralateral mammary gland (inclusive lymph node) was fixed in formalin (for possible later study in a PhD project). Mammary tissue from the offspring will be analysed as a part of another project and reported elsewhere.

The right or left testis was alternately fixed in Bouin's fixative, paraffin embedded and stained with hematoxylin and eosin. The other testis was frozen in liquid nitrogen for hormone analysis (if relevant). The epididymis not applied for motility analysis was excised and weighed.

The following organs were fixed in formalin and subsequent embedded in paraffin: caput epididymis, ventral prostrate, seminal vesicles and a specimen taken from the liver. The remaining part of the liver was frozen in liquid nitrogen for epigenetic analysis as a part of another project that will be reported elsewhere. One section per organ was stained with hematoxylin and eosin for histological evaluation by an examiner blinded to treatment groups. Histopathological evaluation was made of testes, prostate, seminal vesicle and liver from all groups.
In prostate the degree of epithelial atrophy, epithelial infolding and inflammation was scored in the following way: Epithelial atrophy: Score 0: no or minimal epithelial atrophy (≤ 5 acini affected); Score 1: moderate atrophy (> 5% and < 50% of the section affected); Score 2: marked atrophy (≥ 50% of the section affected). Epithelial infolding: Score 1: mild; Score 2: moderate; Score 3: marked. Inflammation: Score 1: no or very few scattered interstitial mononuclear cells; Score 2: focal to few multifocal interstitial accumulations of few mononuclear cells; Score 3: multifocal interstitial accumulations of mononuclear cells; Score 4: diffuse interstitial infiltrations of mononuclear cells.

3.2.5.10 Hormone analysis

Progesterone, testosterone, and estradiol levels were analyzed in rat plasma at PD 13 in the 2nd range-finding study, and for the extended mixture study in the pups at PD 16, PD50/51 and in the dams at PD 22. T4 was measured in male and female pups at PD50. The progesterone levels were analyzed in plasma from 1-5 male and 1-3 female pups in 4-5 litters per dose group. Testosterone and estradiol were analysed in plasma from 1-3 male or 1-3 female pups in 3-5 litters, respectively. Plasma from the pups in each litter

were pooled by sex. Testosterone, estradiol, and progesterone was extracted from the plasma on IST Isolute C18 SPE columns as previously described (Vinggaard ***et al.***, 2005b) and samples were resuspended in heptanes. All hormones including T4 were analysed using Delfia time-resolved fluorescence kits (PerkinElmer Life Sciences, Turku, Finland), and measured by use of a Wallac Victor 1420 multilable counter (PerkinElmer Life Sciences, Turku, Finland).

3.2.5.11 Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall-Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. Birth weights were analysed using the number of offspring per litter as covariate and AGD and organ weights were analysed using body weight as a covariate. Additionally AGD-index (AGDI), namely, AGD divided by the cube root of body weight, was analyzed. The cube root was used because this converts a three-dimensional end point (weight) into a one-dimensional such as the AGD (Gallavan et al., 1999).

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

The number of nipple/areolas was assumed to follow a binomial distribution with a response range between 0 and θ_{max}, with θ_{max} being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θ_{max} was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method as in Hass et al. (2007). The number of nipples in the all control group males was zero and it was therefore necessary to put in 1 nipple in 3 pups from the control group to perform the statistical model.All statistical analysis was performed using the SAS procedure PROC GENMOD.

The statistics on the Morris maze data (i.e. swim length, swim speed and latencies) were calculated for each separate test day, and for the combined total swim length and total latency, which equals a 'repeated measures' test of these endpoints.

The data were analyzed both with all 14 groups, and because of the small group size in groups 5-14, data were also analyzed with only nine groups (pairing the two doses of each pesticide in a single group).

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

Prostate and thyroid histology was analyzed statistically by Fisher's exact test. Mixture groups as well as single compound groups were compared with the control group. In addition some endpoints in prostate were compared between mated and not mated adult rats. In case of statistically significant difference ($p<0.05$) individual scores were compared separately.

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

3.3 Results

3.3.1 Range-finding study in non-pregnant female animals

No signs of clinical toxicity were observed and no effects on body weights were recorded during the first 3 days of the daily dosing. On day 4, clear signs of neurotoxic effects were observed in the animals around 30 min after the dosing with 200 mg/kg and especially 250 mg/kg. The signs observed included immobilization and hind limb paralysis and was scored using a score from 0 to 3, i.e.: Score 1: normal; Score 2: minor signs; Score 3: moderate effect; Score 4: marked effect. The mean scores for the four groups are shown in figure 3.2.

Animals with neurotoxicity score 3 was seen in the highest dose group in 5 of the 6 animals and these animals were sacrificed for animal welfare reasons. The last animal in this group had a score 2 and was kept. In the animals dosed with 200 mg/kg, 1 animal had a score 2 and another had a score 1. All animals dosed with 150 mg/kg had a score 0, i.e. no signs of neurotoxicity. On day 5 before dosing, all animals had a score 0. Based on this, the dose level was lowered to 150 mg/kg on day 5 for the 7 animals dosed with 200 mg/kg (6 animals) or 250 mg/kg (1 animal) on day 1 to 4. After dosing on day 5, however, signs of neurotoxicity effects were observed in these 7 animals. This was in contrast to the animals doses with 150 mg/kg from day 1 to 5 and implied that the 7 animals had some carry-over effect from the previous dosing although they appeared normal. Consequently, these 7 animals were not dosed for 2 days and thereafter dosed with 150 mg/kg. No signs of neurotoxicity were observed during the rest of 22 days dosing period.

The body weight gain was 5-12 g lower in the animals dosed with the pesticides compared to controls (figure 3.3). This effect was considered relatively small because final body weights were only decreased 3-5% and did not differ significantly from the control group.

Overall, the study showed, quite unexpectedly, neurotoxicity in adult animals exposed to a combination of the 5 pesticides at dose levels around their individual NOAELs for effect on parturition. The neurotoxic effect cannot be explained by dose-addition as only mancozeb has shown this effect at higher doses. A possible explanation is that the exposure to the 4 other pesticides may have decreased the excretion of mancozeb and thereby lead to higher blood levels of mancozeb.

3.3.2 Range-finding study 1 in pregnant female animals

No signs of clinical toxicity were observed and no effects on body weight were recorded during the first 3 days of the daily dosing (GD 7-9). On day 4 (GD 10), clear signs of neurotoxic effects were observed in the animals around 30 min after dosing, mainly after the dosing with 218.75 mg/kg. The signs observed included immobilization and hind limb paralysis and was scored using a score from 0 to 3, i.e.: Score 1: normal; Score 2: minor signs; Score 3: moderate effect; Score 4: marked effect. The mean scores for the four groups are shown in figure 3.4. At 218.75 mg/kg, score 2 was seen in 7 of the 8 animals and score 3 was seen for 1 of the 8 animals. At 175 mg/kg, 2 animals was given score 1. All other animals had a score 0, i.e. showed no signs of neurotoxicity.

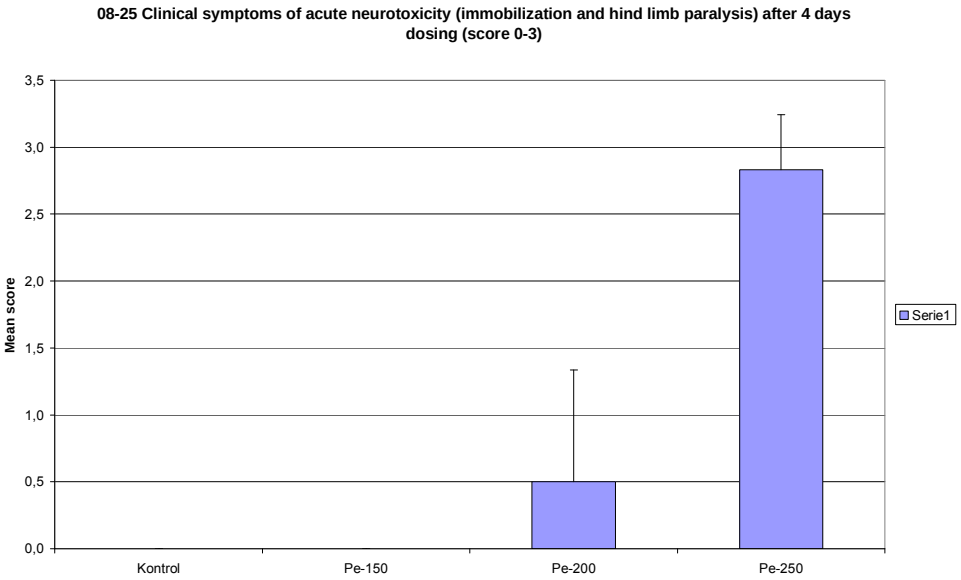


Figure 3.2 Range-finding study in non-pregnant animals. Neurotoxicity scores in non-pregnant females after 4 days of dosing with a mixture of 5 pesticides, mean+std.

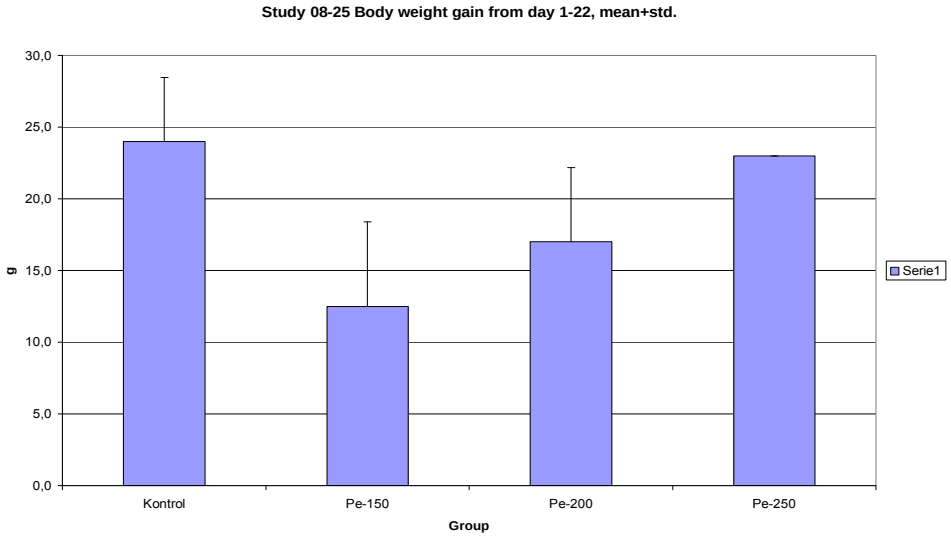


Figure 3.3 Range-finding study in non-pregnant animals. Body weight gain in non-pregnant females after 22 days of dosing with a mixture of 5 pesticides, mean+std. There is only 1 animal in group Pe-250 and consequently no std.

On day 5 (GD 11) before dosing, all animals had a score 0. Based on these findings, the dose level was lowered to 175 mg/kg on day 5 (GD 11) for the animals previously dosed with 218.75 mg/kg. After dosing on day 5 (GD 11), however, signs of neurotoxicity effects were observed in the animal that had a score 3 the day before. Consequently, this animal were not dosed for 2 days and thereafter dosed with 175 mg/kg. No signs of neurotoxicity were observed during the rest of the dosing period.

The body weight gain from GD 10-21 was generally lower in the pregnant animals dosed with the pesticides compared to controls (figure 3.5). The gestation length was clearly affected by the combined pesticide exposure (figure 3.6). All control animals gave birth at the expected time (GD23),

whereas only 1 exposed animal gave birth on GD 23. The remainder of the exposed animals either gave birth on GD24-25 or were sacrificed for animal welfare reasons on GD25 with severe signs of dystochia (problems with giving birth). Caesarean sections of the sacrificed dams showed a high frequency of fully developed foetuses that were either dead or dying.

The number of implantations in the sacrificed animals and implantations scars in the animals giving birth was not affected by the exposure. Together with the high frequency of fully developed foetuses this implies that the effect on the foetuses was induced during the last days of the pregnancy. The percentage perinatal mortality in foetuses and newborn pups was markedly increased in the groups exposed the pesticides compared to controls (87-100% compared to 2.8%).

Overall, the findings from this range-finding study showed clearly that combined exposure to the 5 pesticides at doses around their individual NOAELs for effect on parturition induced severe effects manifested as dystochia and perinatal mortality.

The results from this range-finding study showed that dose levels around the NOAELs for the individual pesticides were much too high for the extended mixture study. Therefore, a new range-finding study using doses significantly lower than the NOAELs for the individual pesticides was needed before planning the extended mixture study.

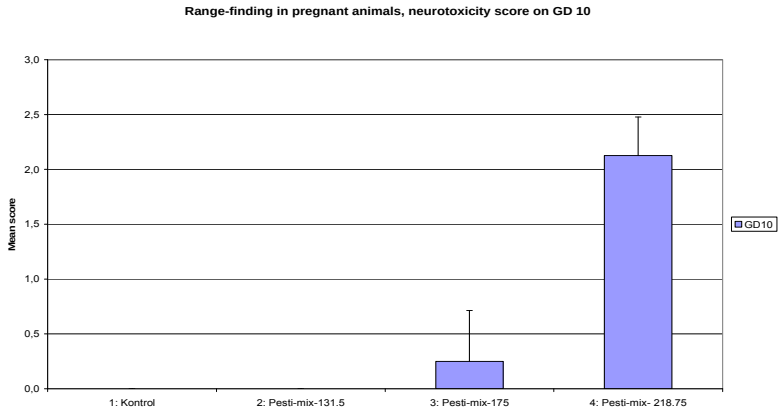


Figure 3.4 Range-finding study 1 in pregnant females. Neurotoxicity score in pregnant females after 4 days of dosing with a mixture of 5 pesticides, mean+std

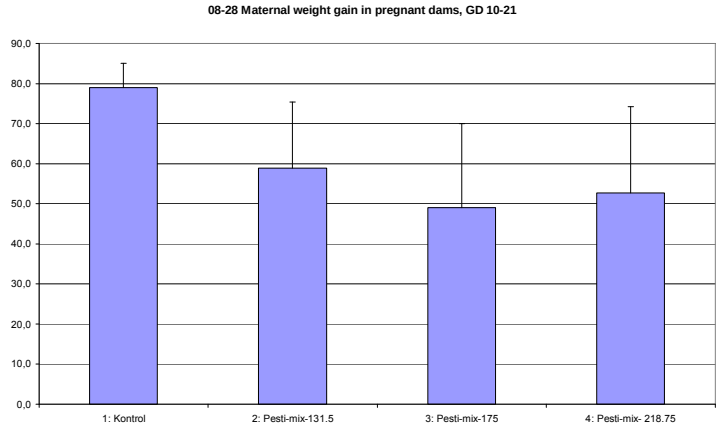


Figure 3.5 Range-finding study 1 in pregnant females. Body weight gain GD 10-21 in pregnant females after dosing with a mixture of 5 pesticides on GD 7-21, mean+std

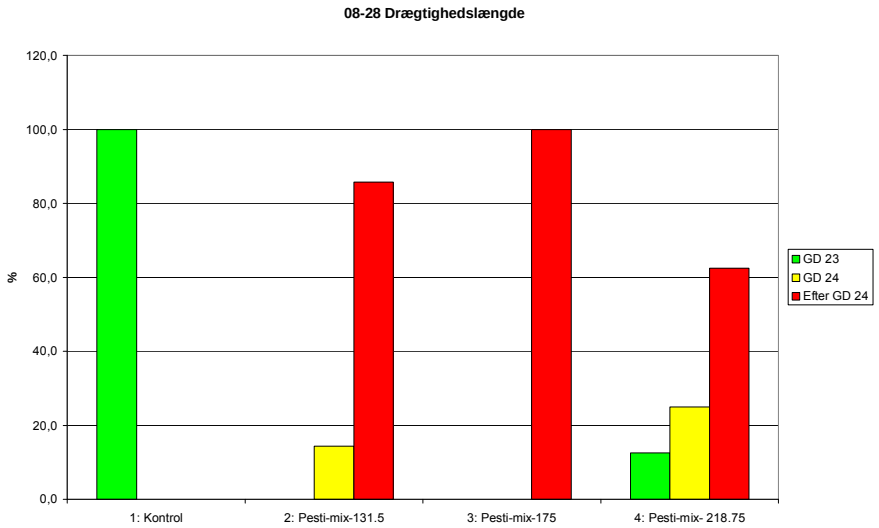


Figure 3.6 Range-finding study 1 in pregnant females. Gestation lengths in pregnant females after dosing with a mixture of 5 pesticides on GD 7-21, % distribution

3.3.3 Range-finding 2 in pregnant female animals

The dose levels of the pesticide mixture (mix) were 43.75 and 87.5 mg/kg bw/day, i.e. 25% and 50% of the initial mixture dose. The results of this study have been analyzed and evaluated together with the results from the first range-finding study and a paper has been published (Jacobsen et al., 2010). The first range-finding study in pregnant animals used dose levels of 131.25 mg/kg bw/day (mix-75%), 175.0 mg/kg bw/day (mix-100%) and 218.8 mg/kg bw/day (mix-125%). As described earlier, the dams dosed with of mix-125% exhibited signs of acute neurotoxicity after two days of dosing and consequently, the dose was decreased to mix-100% from the third day of dosing. The results from this group were in the combined analysis of the two range-finding studies included in the mix-100% group.

3.3.3.1 Pregnancy data and postnatal survival

There were no statistically significant effects on maternal body weight gain from GD 7-GD 21 and GD 7-PD 1 in dams exposed to mix-75% or lower (table 3.7). However, maternal body weight gain from GD 7-GD 21 in dams exposed to the highest dose of the mixture (mix-100%) was significantly decreased (table 3.7).
Gestation length was significantly increased in all dosed groups (table 3.7) and 5 of 7 dams in mix-75% and 9 of 14 dams in mix-100% were unable to give birth and had to be sacrificed on GD 25 (fig. 3.7a).
The number of liveborn pups was significantly decreased and the perinatal pup loss was significantly increased at mix-50% and higher when compared to controls (table 3.7, fig. 3.7b). No effects on birth weight were observed in male pups compared to controls, whereas the female pups exposed to mix-50% had a significantly decreased birth weight (table 3.7).

3.3.3.2 AGD and NR

It was only possible to record AGD in a few litters in the first range-finding study as most of the dams were unable to give birth (data not shown). In the second study the mixture produced dose-dependent changes in AGD index (AGI) with a statistically significant increase seen in females and a decrease in males (fig. 3.8a). Nipple retention was significantly and dose-dependently

increased in male pups in both groups exposed to the mixture i.e. mix-25% and mix-50% (fig. 3.8 b).

3.3.3.3 Autopsy PD 13, organ weight and genital dysgenesis

No effects were observed on weight of the testes or the uterus in male and female offspring, respectively. Weights of prostate and epididymis in male pups were decreased in mix-25% and mix-50% exposed animals (table 3.7). The liver weights of both male and female pups were elevated in the mix-50%-treated animals, but no effects were observed on liver weights of the dams (table 3.7).

The incidence of genital dysgenesis was increased with increasing dose (fig. 3.8c). In the mix-25%, the males had either no, mild or moderate dysgenesis (score 0-2), whereas all of the males in the mix-50% group showed severe dysgenesis of the genitalia (score 3). No animals in the control group showed any malformations.

Table 3.7 Range-finding study 1 and 2 in pregnant animals. Pregnancy and weight data. Orgqan weights are on pup day 13. Data represent group means, based on litter means ± SEM

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

* P<0.05, **P<0.01 and *** P <0.0001. Birth weight was analysed using the number of offspring as a covariate. Organ weights PD 13 were analysed using body weight as a covariate - No data because of caesarean section ; a) Study 1; b) Study 2; a+b) The control group is representing both study 1 and 2

3.3.3.4 Hormone levels

No statistically significant effects of exposure to mix (25%, 50%) on progesterone, testosterone or estradiol serum levels were revealed in dams or in male and female pups (table 3.8). However, the biological variation in

combination with the small number of samples makes it possible to detect only very pronounced effects.

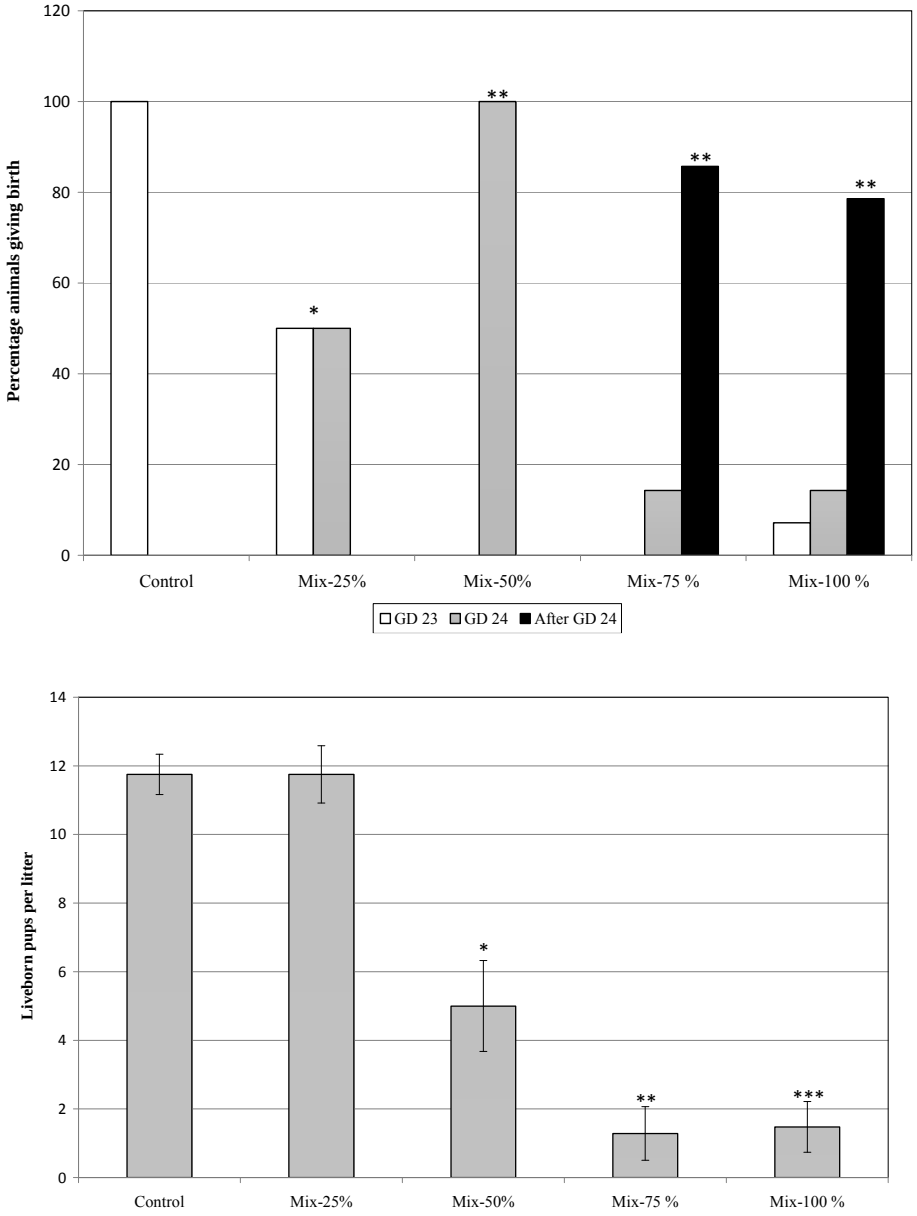
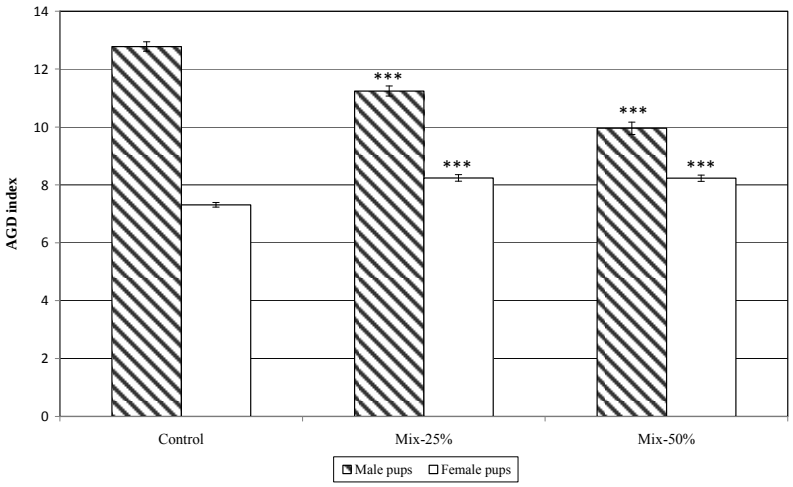
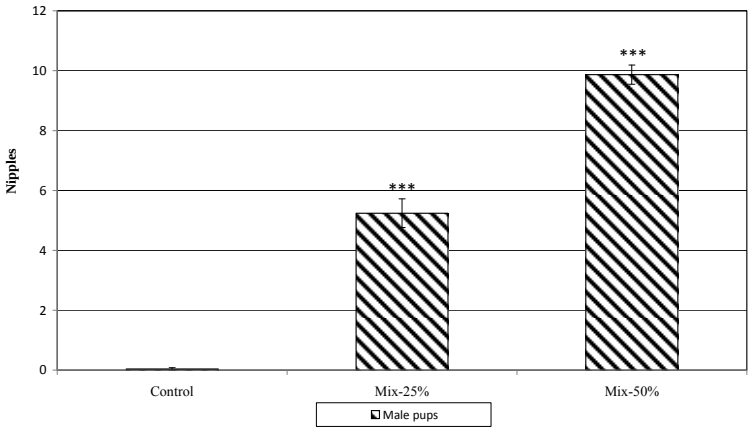


Figure 3.7 a, b. Range-finding study 1 and 2 in pregnant females. Effects of combined exposure to procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb on day of parturition (a.) and live litter size in rats (b.). The parturition results are shown as percentage animals giving birth on gestation days 23, 24 and after day 24. A few of the animals in the latter group gave birth on gestation day 25, but most of them were unable to give birth. Results shown for number of liveborn pups per litter are group mean ± standard deviation. p-values are * < 0.05, ** < 0.01 and *** <0.001. N = 8(94), 4(47), 7(40), 7(9) and 14(22) litters (liveborn pups) in Control, Mix-25%, Mix-50%, Mix-75% and Mix-100% respectively.





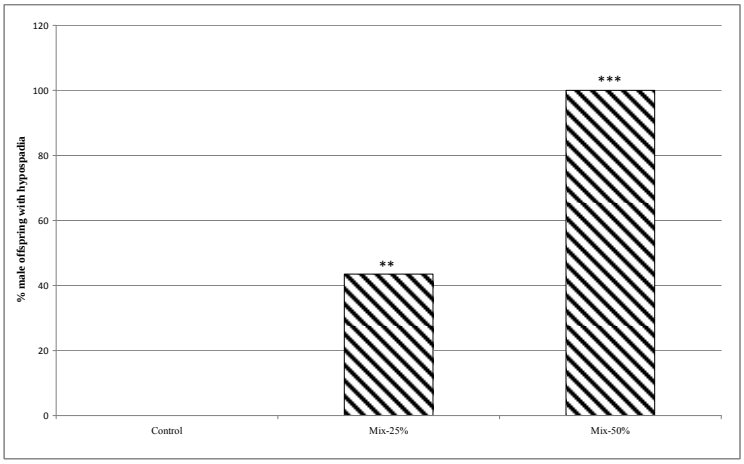


Figure 3.8 a, b,c. Range-finding study 1 and 2 in pregnant females. Effects of combined exposure to procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb on anogenital distance (AGD) index at birth (a), nipple retention on pup day 13 (b) and genital dysgenesis on pup day 13 (c). Results shown are group mean ± standard deviation. The number of nipples in female controls is generally 12. See text for details on the endpoints. p-values are * < 0.05, ** < 0.01 and *** <0.001. N = 8(45:49), 4(26:21), and 7(24:15) litters (male:female pups) in Control, Mix-25% and mix-50%, respectively.

Table 3.8 Range-finding study 1 and 2 in pregnant animals. Hormone levels in dams and pups. Data represent group means, based on pooled serum ± SD, N= 3-5 litters in each group.

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

3.3.4 Results extended mixture study

3.3.4.1 Pregnancy, litter and offspring data

Pregnancy and litter data are shown in table 3.9 in Appendix 1. There were no statistically significant effects on maternal body weight gain from GD 7-GD 21 and from GD 7-PD 1 in exposed dams compared to control dams. For the mixture, this is consistent with the results in range-finding study 2, where maternal body weight gain was only affected at much higher doses of the mixture. No clinical signs of toxicity were observed in the dams and the number of implantation scars in the uterus, postimplantational- and perinatal loss was similar among groups.

Gestation length was significantly increased in the two highest mixture groups (Group 3, 4) and in the group exposed to the highest dose of epoxiconazole (group 6), but not in any other groups. These results show combination effect of the 5 pesticides at dose levels where the individual pesticides caused no effects. Increased gestation length of very similar magnitude was also observed in range-finding study 2 in the group exposed similarly as group 4 in this study.

Offspring data are shown in table 3.10 in Appendix 1. There were no effects on pup body weights at birth or on PD 6, 13 and 22. For the mixture, this is consistent with the results in range-finding study 2, where pup birth weight was only affected at a two higher dose of the mixture and only in female offspring.

Nipple retention in male offspring was significantly higher than controls in all mixture groups, at the highest dose of mancozeb, prochloraz and tebuconazole and both doses of procymidone (fig. 3.9). For the highest mixture group, the result is consistent with the result in range-finding study 2 in the group exposed similarly as in this study. Since the magnitude of the effect in the highest mixture group (group 4) is statistically significantly higher that the effect induced by procymidone alone at the dose level included in the highest mixture (group 13), ($p<0.001$) these results show mixture effects for this endpoint.

In the male offspring, no statistically significant effects on anogenital distance (AGD) were seen in the mixture groups. At the dose levels included in the mixture for the individual chemicals, epoxiconazole induced longer AGD, whereas procymidone induced shorter AGD. Consequently, the lack of effect in the mixture group might be due to two chemical influences going in opposite directions.

In the female offspring, significantly longer AGD index (AGDI), i.e. AGD/cubic root of body weight, was seen in the two highest mixture groups (groups 3 and 4). A similar effect was seen in animals exposed to both doses of prochloraz and tebuconazol and in the group exposed to the low dose of epoxiconazol but not in animals dosed with a four times higher dose.(table 3.10 in Appendix 1) Therefore, the effect in the mixture groups may or may not be a combination effect. Further evaluation of this is included in the chapter on modelling of the mixture effects (chapter 4).

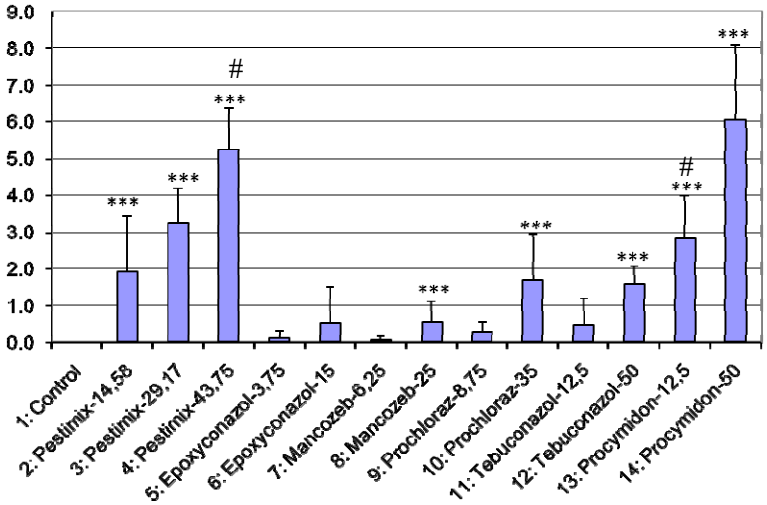


Figure 3.9. Extended mixture study. Effects of single and combined exposure to procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb on nipple retention in male pups PD 13. Results shown are group means ± standard deviation. See table 3.10 in appendix 1 for more details on results. The number of nipples in female controls is normally 12. p-values are *** <0.001. # The difference between group 4 and 13 is statistically significant (p<0.001).

3.3.4.2 Section PD 16, organ weights

Uterus, ovary, liver and body weights of females are shown in table 3.11 in Appendix 1. No statistically significant changes in body or organ weights were observed.

Absolute and relative weights of male reproductive organs are listed in table 3.12 A and 3.12 B, respectively, in Appendix 1. Reduced absolute weights of epididymis, prostate and seminal vesicle were seen in the highest mixture dose group compared to controls, when including body weight as a covariate in the statistical analysis in a model using Dunnett's post hoc test on all 14 dose groups (marked with * in the tables). Epididymis weight was lower than controls in all three mixture groups though this was only statistically significant in group 2 and 4 (low and high mixture groups).

Additionally, a statistically significant increase in absolute prostate weight was seen in animals exposed to the highest dose of epoxiconazole. In a previous study on epoxiconazole in our lab, a tendency to increased weights of male reproductive organs including prostates was seen, but only a few (1-2) animals were available for analysis in that study (Taxvig et al., 2007). Dam testosterone levels were increased during gestation in the epoxiconazole treated animals in that study and this may have had a growth promoting effect.

Weights of testes, levator ani/bulbocavernosus muscle (LABC), glandula bulbourethralis, liver and thyroid were not significantly different between groups. Relative weights of epididymis, prostate and seminal vesicle were significantly reduced in the highest mixture dose group compared to controls, whereas an apparent increase in relative prostate weight in the high dose epoxiconazole group was not statistically significant. A statistically significant increase in testis weights was observed in animals exposed to the lowest dose of procymidone. Interestingly, our own previous study on procymidone showed a statistically significant increase in testis weight at 10 mg/kg bw/day, no change at 25 to 100 mg/kg bw/day and a decrease at 150 mg/kg bw/day (Metzdorff et al., 2007). No other statistically significant changes in male relative organ weights were observed.

The reductions in weights of male accessory reproductive organs in only the highest mixture group indicates a "something from nothing" type of effect, i.e. that no statistically significant changes were seen for the single compounds at the doses used in the mixture. It should be noted here, that the lowest doses of the individual compounds are similar to the doses used in the highest mixture group. As the numbers of animals in the individual chemical groups were lower than the number of animals in the mixture groups, it may be argued that the low "n" of the individual chemical groups may reduce the likelihood for detecting statistically significant changes. However, by pooling weight data from the low and the high dose groups for each chemical the "n" was increased to 9-13 litters per chemical group. Even then, no statistically significant changes could be observed between controls and individual chemical groups, except for an increase in testis weight in the pooled procymidone group. Thus, increasing the number of litters in the individual chemical low dose groups would not change the overall picture of a "something from nothing" effect on male reproductive organ weights.

As described in the statistics section, Dunnett's test corrects for multiple comparisons, but applying this test on a study with 14 groups may lead to over-compensation and may lead to false negative results. We therefore also applied an alternative approach using a Dunnett's post hoc test on each group of chemicals separately (mixture groups 2-4 and single compounds) and marked the results with # in the tables.

When statistics were performed using a Dunnett's post hoc test on each group of chemicals separately, statistically significant changes in organ weights were also seen for high doses of some of the individual pesticides. The high dose of epoxiconazole increased epididymis and prostate weights, the high dose of prochloraz decreased epididymis and thyroid weights, and the high dose of procymidone reduced weights of epididymis, prostate and the bulbo urethral gland. Also relative weights of bulbourethral glands were significantly reduced by procymidone using this statistical approach. Similar reductions of male reproductive organ weights have been seen from 10 or 25 mg/kg bw/day of procymidone in our previous study (Metzdorff et al., 2007). As these effects on androgen sensitive organs were seen in the high but not the low dose groups (applied in the high mixture), the impression of a "something from nothing" effect holds true also using this statistical approach.

Paired testis weight, on the other hand, was significantly increased in two highest mixture groups and the low dose procymidone group, and if this is indeed a true effect, it may show the effect of the procymidone content in the

mixture on testis development. Further studies on PD 16 testes are described in the histology section. The observed reduction of thyroid weights by prochloraz has not been described in the literature and has not been investigated in our previous studies on prochloraz.
Furthermore, relative and absolute liver weights were increased in the tebuconazole high dose group (50 mg/kg bw/day) compared to controls. This confirms findings in a study by Moser et al., 2001, describing increased liver weights on PD 46 in rats following perinatal exposure to 60 mg/kg bw/day of tebuconazol.

3.3.4.3 Section on PD 16, histology

Thyroid weights in male offspring were not altered by exposure (see table 3.12 A and 3.12 B in appendix 1). However, due to the small size of this organ on PD 16, changes in organ weights may be difficult to detect. Additionally, structural changes indicative of disturbed development of thyroids may be visible by histological evaluation but not reflected as changes in organ weights. Thyroid histology (control and all three mixture groups) was evaluated in female offspring, and no clear differences between groups were observed (table 3.13). A few animals from all dose groups had larger, cuboidal epithelial cells in contrast to the more flattened (squamous) epithelium seen in most controls. The cuboidal epithelium may reflect a smaller amount of follicular fluid (colloid) in lumini and thus reduced function. The number of animals with this altered appearance of thyroids was not statistically significant between groups (Fisher's exact test).

Table 3.13.Extended mixture study. Thyroid histology in female pups PD 16

Thyroid histology, PD 16 females	Number of animals	Mainly squamous epithelium	Presence of cuboidal epithelium	Presence of columnar epithelium	Slight hypertrofia or/and hyperplasia	Presence of irregular follicles	Increased amount of connective tissue
1: Control	10	100% (10/10)	30% (3/10)	0% (0/10)	20% (2/10)	10% (1/10)	60% (6/10)
2: Pestimix - 14,58	9	78% (7/9)	67% (6/9)	11% (1/9)	67% (6/9)	56% (5/9)	33% (3/9)
3: Pestimix - 29,17	7	86% (6/7)	43% (3/7)	14% (1/7)	57% (4/7)	14% (1/7)	57% (4/7)
4: Pestimix - 43,75	10	80% (8/10)	70% (7/10)	0% (0/10)	40% (4/10)	40% (4/10)	60% (6/10)

Table 3.14 Extended mixture study. Sem. vesicle histology in male pups PD16

Seminal vesicle histology, PD 16	No. of animals evaluated	Hypoplasia	Areas of structural dysorganization
1: Control	13	8% (1/13)	8% (1/13)
2: Pestimix-14,58	14	6% (1/14)	7% (1/14)
3: Pestimix-29,17	7		
4: Pestimix-43,75	8		25% (2/8)
5: Epoxi-3,75	6		17% (1/6)
6: Epoxi-15	3		
7: Mancozeb-6,25	5		
8: Mancozeb-25	7		
9: Prochloraz-8,75	8		13% (1/8)
10: Prochloraz-35	4		25% (1/4)

11: Tebu-12,5	5	13% (1/5)	40% (2/5)
12: Tebu-50	5		
13: Procy-12,5	6		
14: Procy-50	3		

Seminal vesicle weight was significantly reduced in the highest mixture group, but no clear alterations were observed in the histological examination (see table 3.14). A large degree of variation was seen due to the irregularity of this organ.
Epididymis weight was significantly reduced in the highest mixture group, but no clear histological alterations were observed (table 3.15).

Table 3.15 Extended mixture study. Epididymis histology in male pups PD 16

Epididymis histology, PD 16	No. animals	Presence of area with dark nuclei	Presence of area with light nuclei	Sloughed cells in lumen
1: Control	15		7% (1/15)	7% (1/15)
2: Pestimix-14,58	16			
3: Pestimix-29,17	8	13% (1/8)	13% (1/8)	13% (1/8)
4: Pestimix-43,75	12		8% (1/12)	
5: Epoxiconazol-3,75	6	17% (1/6)		
6: Epoxiconazol-15	3			
7: Mancozeb-6,25	5			
8: Mancozeb-25	7	14% (1/7)		14% (1/7)
9: Prochloraz-8,75	9		11% (1/9)	
10: Prochloraz-35	4		25% (1/4)	50% (2/4)
11: Tebuconazol-12,5	8			25% (2/8)
12: Tebuconazol-50	5			
13: Procymidon-12,5	6			
14: Procymidon-50	3			

Testis histology at PD 16 was examined by a semi-quantitative assessment of seminiferous tubule formation. This was carried out by measuring tubular diameter and evaluating lumen formation (percentage of tubule cross-sections with lumen) in 100-120 randomly selected seminiferous tubules per testis.

The evaluation of lumen formation showed a high degree of variation in the control group, and no statistically significant differences between controls and dose groups were observed (fig. 3.10). A statistically significant correlation between lumen formation and seminiferous tubule diameter was observed, indicating that testes with large tubule diameters also have a high degree of lumen formation in seminiferous tubules. However, this is not evident when comparing group mean values for lumen formation and tubule diameter and due to the large variation in data, lumen formation cannot be considered a sensitive measure of testis development.

Statistically significant reductions in seminiferous tubule diameters were seen in group 2 and 8 compared to controls when applying a Dunnett's test for all 14 groups (fig. 3.11). In a comparison of each type of pesticide or mixture to controls separately, statistically significant reductions in seminiferous tubule diameter were seen in group 2, 5, 8, 11, 12, 13. As some of these groups are low dose groups and no effect is seen in the corresponding high dose groups, this may not be dose-related effects. The reduction in tubule diameter in

group 8 (high dose mancozeb) corresponded to a slight (not statistically significant) decrease in testis weight in that group and may be considered dose related.

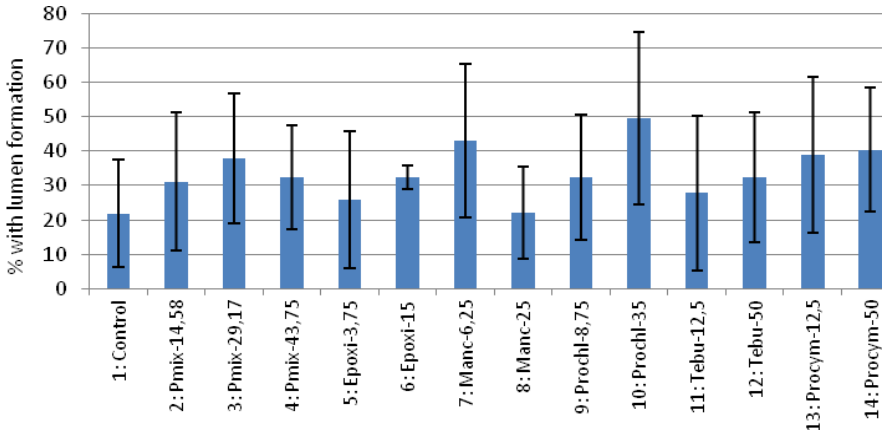


Figure 3.10 Extended mixture study. Histopathological effects in testes of male offspring PD 16. % males with lumen formation in seminiferous tubules. Results shown are group means ± SD.

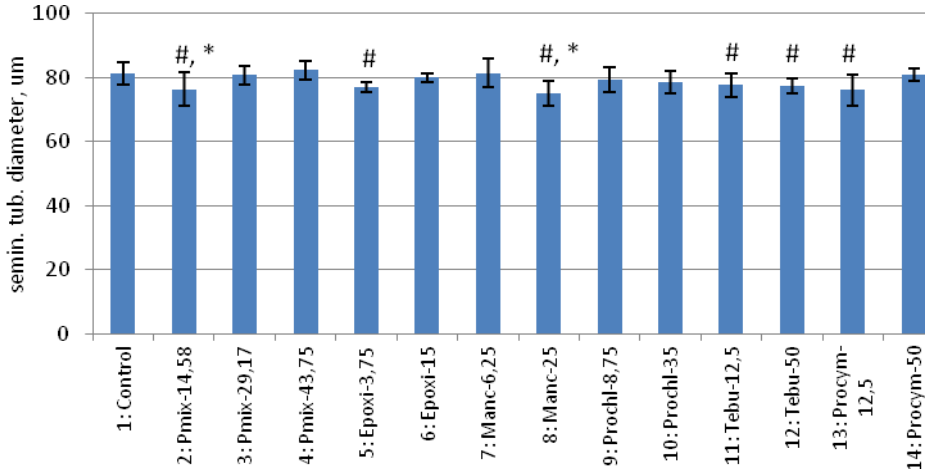


Figure 3.11 Extended mixture study. Histopathological effects in testes of male offspring PD 16. Seminiferous tubule diameter. Results shown are group means ± SD.

As described in the section on organ weights, paired testis weight was increased in group 13 and possibly group 3 and 4, which is in contrast to the apparent delay in testis development evidenced by decreased tubule diameters in groups 2 and 8 and possibly 5, 11, 12 and 13. However, correlation analysis showed statistically significant correlations between testis weight and lumen formation and between testis weight and seminiferous tubule diameter (i.e. testes with high absolute weights have large tubule diameters and a high percentage of lumina). It may be interesting to know whether measurement of seminiferous tubule diameters is a sensitive indicator of delayed testicular development and whether this is more sensitive than testis weight at this age, but this would need to be examined further in other studies on chemicals known to delay testis development.

3.3.4.4 Section adult offspring, organ weights

Absolute and relative weights of uterus, ovary and liver of females are shown in table 3.16 in Appendix 1. No statistically significant changes in female body or organ weights were observed.

Absolute and relative weights of male reproductive organs are listed in table 3.17 A and 3.17 B, respectively in Appendix 1. Body weight appeared slightly reduced in most dose groups though this was not statistically significant in the statistical approach using Dunnett's test on all 14 groups. When separating the dataset into single compound groups the reduction in body weight was statistically significant in group 2, 6, 10 and 11 (low mixture group, high epoxiconazol, high prochloraz, low tebuconazol).
Reduced absolute weight of LABC and prostate were seen in the highest mixture dose group (group 4) compared to controls. In the group exposed to the high dose of procymidone (group 14), prostate and liver weights were significantly reduced compared to controls.

The decreased weights of prostates in the high mixture group showing that pesticide effects on male reproductive organs are persistent corresponds well with the finding of low weights of prostates, seminal vesicles and epididymides on PD 16. No changes in seminal vesicle or epididymis weight was observed in adult animals, indicating that PD 16 may be a more sensitive time point for observing impaired growth of male reproductive organs due to anti-androgenic effects of these pesticides. In contrast, LABC weights were reduced only in adulthood, indicating that the anti-androgenic influence on this organ is persistent. Our own previous studies on perinatal exposure to other anti-androgenic chemicals have shown that prostate weight on PD 16 is often affected at low doses. For different anti-androgenic chemicals, it differs whether LABC, seminal vesicle or epididymis weights are also affected at the same doses that affect prostate weight, i.e. prostates appear to be most sensitive whereas weights of other male reproductive organs vary in their sensitivity depending on the type of chemical exposure.

The low body weight observed in mainly the tebuconazole groups resulted in increased relative, but not absolute, weights of some organs (increased relative testis weight in group 5 and 6; increased relative weights of epididymis, bulbourethral gland and thyroids in group 6). As these increases were only seen for relative weights and not for absolute weights with body weights as covariate, these effects are considered to be due to body weight changes rather than to direct effects of pesticide exposure on target organs. Furthermore it should be noted that group 6 only consisted of 2 adult males and that results therefore should be interpreted with caution.
Statistical analysis of relative organ weights also revealed significantly decreased relative liver weight in group 10 and 14, whereas absolute liver weights analyzed with body weight as a covariate were not significantly decreased in group 10, but in group 14. This points to dose related effects of the high doses of prochloraz (group 10) and procymidone (group 14).

Overall, thyroid weights were not affected by treatment. However, it may be noted that standard deviations were increased in some groups reflecting that one animal from each of the mancozeb groups and one animal from the low dose mixture group had more than double thyroid weight compared to controls and to other pesticide exposed animals.

As also described for PD 16, the reduction in weights of male accessory reproductive organs (prostate and LABC) in only the highest mixture group indicates a "something from nothing" type of effect, i.e. that no statistically significant changes were seen for the single compounds at the doses used in the mixture. It should be noted that the lowest doses of the individual compounds are similar to the doses used in the highest mixture group.

3.3.4.5 Section adult offspring, malformations

Malformations related to the reproductive system and seminal vesicles were observed in a few animals (table 3.18). Chryptochidism and alopecia in the perineal area were not observed in any of the adult males. Two males in the highest mixture group had penile malformation: one had both a severe cleft penis and hypospadia with urethral opening at the basis of the penis (score 3), the other had a cleft phallus (score 1). A third male had a malformed seminal vesicle. One male in each of the groups dosed with mancozeb showed malformation of the seminal vesicle. In the group dosed with the highest level of procymidone two animals had cleft phallus (score 2 and 3, respectively), and a third animal had malformed seminal vesicle.

Table 3.18 Extended mixture study. Genital malformations (cleft phallus and hypospadia) and malformations of seminal vesicles in adult male rats observed at autopsy.

	Number of animals	Number of animals with penile malformations	Number of animals with malformed seminal vesicles
1: Control	16	0	0
2: Pestimix-14,58	18	0	0
3: Pestimix-29,17	12	0	0
4: Pestimix-43,75	16	2	1
5: Epoxiconazol-3,75	10	0	0
6: Epoxiconazol-15	2	0	0
7: Mancozeb-6,25	8	0	1
8: Mancozeb-25	9	0	1
9: Prochloraz-8,75	10	0	0
10: Prochloraz-35	6	0	0
11: Tebuconazol-12,5	9	0	0
12: Tebuconazol-50	8	0	0
13: Procymidone-12,5	8	0	0
14: Procymidone-50	6	2	1

3.3.4.6 Section adult offspring, histology

Testicular histology appeared normal in most animals, whereas two animals had testes with tubular degeneration in approximately 20% of seminiferous tubule cross-sections. As this finding may also be seen in controls and as these animals were from two different low dose groups (tebuconazole and procymidone), this was not considered to be dose related.

In prostate, various lesions were observed in both controls and exposed animals. Interstitial inflammation and intraepithelial vacuolation was observed in all groups with no statistically significant differences between exposed groups and the control group (table 3.19 in appendix 1). Additionally acini with concretions in the lumen and focal or multifocal areas with few acini surrounded by increased amount of stroma and lined by atrophic epithelium were found in a large number of animals in all groups (data not presented). Histological sections containing small amount of prostate tissue were observed from 6% of rats in group 2, 19% of rats in group 4 and 25% of rats in group 7. Those six sections were from the animals with the top ten lowest absolute and relative prostate weights indicating atrophy of the prostate. In all evaluated histological sections areas with multilayering of epithelial cells were observed,

and although no clear differences was observed between the groups (data not presented) it could be investigated more thoroughly in the future. The size of the acini, the height of the epithelium lining the acini, the degree of epithelial infolding and the secretory content in the lumen of acini varied a lot in all groups. Generally acini with marked epithelial infolding were lined by columnar epithelium and had small lumina. In contrast acini with minimal epithelial infolding were generally lined by cuboidal to simple squamous epithelium and appeared dilated (table 3.20 in appendix 1). No statistically significant differences were observed in the dominating epithelial type lining the acini or in the occurrence of focal acinar atrophy (defined as areas with groups of irregular acini lined by simple squamous epithelium and lacking secretory content in acinar lumen and with increased distance between acini) (table 3.19 and 3.20 in appendix 1). However, the number of animals with mild epithelial infolding (score 1) was significantly lower and the number of animals with moderate epithelial infolding (score 2) was significantly higher in group 2 (low dose mixture) and group 9 (low dose prochloraz) compared to the control group (group 1). These findings were not considered dose related, as dose response was not observed.

In group 4 (high dose mixture), significantly less acinar epithelial atrophy was observed compared with the control group (group 1) (table 3.20 in appendix 1). The above results could indicate a lower rate of glandular empyting in control animals resulting in cessation of secretory activity and consequently more dilated acini without epithelial infolding and flatter epithelium.

The great variation in prostate morphology within the groups, the high frequency of lesions in all groups including controls, and the fact that focal epithelial and acinar atrophy are spontaneous changes in rats complicate the interpretation of the results. Additionally the results could be influenced by the fact that both unmated rats and rats mated 1, 2 or 3 days prior to section were included in the study.

A significantly higher number of mated rats had acini mainly lined by columnar epithelium and a significantly lower number of mated rats had acini mainly lined by simple squamous to cuboidal epithelium compared to unmated rats (table 3.21 in appendix 1). In addition marked epithelial infolding was observed in significantly more mated rats than not mated rats, and marked epithelial atrophy (score 2) was observed in significantly fewer mated rates compared to not mated rats (table 3.21 in appendix 1). These results could reflect change in acini morphology secondary to mating. Although the number of mated and not mated rats was similar in most groups including group 2 and 4 the statistically significant differences in epithelial infolding and epithelial atrophy observed in group 2 and 4, respectively, disappear if only not mated animals were compared, and some of the statistically significant differences disappear if only mated animals were compared. In conclusion no clear dose-related differences were found in prostate histopathology.

In the seminal vesicle a large degree of variation in lumen size, epithelial height, epithelial infolding and secretory content were seen. No significant histological lesions were observed in the majority of the animals. However, vesiculitis was observed in three animals (one from group 4 and two from group 14). Minimal inflammatory cell foci were observed in three animals (group 2, 7 and 11). In four animals (two from group 4, one from group 2

and one from group 14) few macrophages containing yellow brown pigment corresponding to hemosiderin were observed in tunica muscularis.

The activity of the thyroid was generally not very marked in the evaluated histological sections. Follicles with high columnar vacuolated epithelial cells indicating higher degree of activity were only observed in few animals, mainly belonging to group 8 (high dose mancozeb) (table 3.22). Correspondingly the number of animals with follicles dominated by columnar to cuboidal epithelium were significantly higher in group 8 compared to the control group (table 3.22). Mancozeb has previously been shown to produce structural and functional changes in thyroid of rats including hyperplasia and hypertrophy of follicular cells (Trivedi et al., 1997, Kackar et al., 1997). Mild diffuse C-cell hyperplasia was observed in all groups with no statistically significant differences. It is known that the extent of C-cell hyperplasia increases with advancing age. Ultimobranchial cysts were found in two animals (group 1 and 8). In one animal from group 8 diffusely vacuolated thyroid follicular epithelial cells with apical located nucleus were observed. Similar intraepithelial vacuolation has been suggested to be related with a genetic factor (Shimoi et al., 2001).

Table 3.22. Extended mixture study. Thyroid histology in adult females. Statistically significant differences between controls and exposed are marked with bold. * indicate p<0.05

Thyroid histology adult females	Number of animals evaluated[$]	Mainly columnar to cuboidal epithelium	Mainly cuboidal epithelium	Mainly simple squamous to cuboidal epithelium	Presence of follicles with high columnar vacuolated epithelial cells	Mild C-cell hyperplasia
1: Control	17	6% 1/17	59% 10/17	35% 6/17	12% 2/17	41% 7/17
4: Pestimix-43,75	11	9% 1/11	82% 9/11	9% 1/11	9% 1/11	36% 4/11
7: Mancoz-6,25	7	14% 1/7	71% 5/7	14% 1/7	29% 2/7	43% 3/7
8: Mancozeb-25	9	44%* 4/9	33% 3/9	22% 2/9	44% 4/9	33% 3/9

[&]One animal was excluded from each group either due to missing tissue (group 1, 4 and 7) or due to severe vacuolation of epithelial cells.

No clear differences were observed in liver histopathology between controls and the exposed groups (table 3.23 in appendix 1). In all animals minimal inflammatory cell infiltrations dominated by mononuclear cells were observed in the portal areas. In a few animals unifocal or few multifocal small parenchymal inflammatory cell infiltrations dominated by mononuclear cells were observed. Additionally few small centrilobular inflammatory cell infiltrations dominated by mononuclear cells were found in few animals. In all groups minimal hepatocyte vacuolation were observed. In two animals moderate amount of light yellow brown pigment corresponding to hemosiderine was observed in macrophages mainly in portal areas and in few periportal hepatocytes. In one animal (group 5, low dose epoxiconazol) a neoplasia corresponding to malignant lymphoma was found.

3.3.4.7 Genital malformations in male offspring, PD 16, 22 and 50

The results of the scoring of external genital malformations in male offspring that were sectioned on PD 16 and 22 are shown in tables 3.24 and 3.25,

respectively. The results at each time points indicate increased number or severity of genital malformation in the highest mixture group and the highest dose of procymidone, but the differences are not statistically significant compared to controls. No animals had increased gubernacular length as an indication of cryptorchidism.

The rest of the male animals were scored for external genital malformations alive shortly after sexual maturation (around PD 50) and the results were combined for all time points (see figure 3.13 and table 3.26). The combined results show increased number and severity of external genital malformation in the highest mixture group and the highest dose of procymidone and these differences are statistically significant compared to controls.

The group mean of the percentage of animals within each litter with external genital malformations is markedly higher in the highest mixture group than in any of the groups dosed with the individual pesticides alone at the dose included in the mixture (around 17% compared to 0-4%, figure 3.12). The individual pesticides alone caused no statistically significant effect at this dose level.

At the same mixture dose, the incidence of genital dysgenesis in the range-finding study 2 was around 40%, whereas the incidence of genital malformations in the extended mixture study was around 17%. This difference may be related to the lower number of litters in the group in range-finding study compared to the group in the extended mixture study, i.e. 4 and 12 litters, respectively. This means that the incidence found in the range-finding study is more uncertain than the incidence found in the extended mixture study. The age where evaluation took place also differs as all male offspring were scored on PD 13 in the range-finding study, whereas the assessment in the extended mixture study was done at later developmental stages, i.e. PD 16, 22 or 50. The external male reproductive organs undergo substantial development after PD 13 and are not fully developed until after sexual maturation. The lower incidence in the extended mixture study could therefore also partly reflect that it is more difficult to score the animal at an early stage. Actually, that was one of our reasons for postponing the preweaning section of the male pups from PD 13 to PD 16 in the extended mixture study.

Overall, the incidence around 17% found in the extended mixture study is considered the most reliable finding.

Table 3.24 Extended mixture study. Genital malformations in male pups sectioned on PD 16. Values indicate the number of animals with score 0 (normal), score 1 (slight penile malformation) or score 2 (moderate penile malformation). No animals had increased gubernacular length as an indication of cryptorchidism. Each animal represents one litter.

Dose group	Total number of animals	Malfor-mation score 0	Malfor-mation score 1	Malfor-mation score 2	Malfor-mation score 1-2	Statistics
1: Control	15	15				
2: Pestimix-14,58	16	16				
3: Pestimix-29,17	8	8				
4: Pestimix-43,75	12	9	2	1	3	NS,p=0.07
5: Epoxicon-3,75	6	6				
6: Epoxico-15	3	3				

7: Manco-6,25	5	5				
8: Manco-25	7	7				
9: Prochlo-8,75	9	9		1	1	NS
10: Prochlo-35	4	3	1		1	NS
11: Tebu-12,5	8	8				
12: Tebu-50	5	5				
13: Procy-12,5		6				
14: Procy-50		2	1		1	NS

Table 3.25 Extended mixture study. Genital malformations in male pups sectioned on PD 22. Values indicate the number of animals with score 0 (normal), score 1 (slight penile malformation), score 2 (moderate penile malformation), or score 3 (severe penile malformation). Values in parentheses are the number of litters with the indicated score. No animals had increased gubernacular length as an indication of cryptorchidism (measured in one male per litter).

Dose group	No. anim. (litters)	Malfor-mation score 0	Malfor-mation score 1	Malfor-mation score 2	Malfor-mation score 3	Malfor-mation score 1-2-3	Statistics
1: Control	30 (10)	31 (10)					
2: Pestimix-14,58	44 (11)	42 (10)	1(1)		1(1)	2 (1)	NS
3: Pestimix-29,17	22 (7)	23 (7)		1(1)			
4: Pestimix-43,75	24 (10)	21 (7)		2(2)	2(2)	4(4)	NS
5: Epoxi-3,75	18 (4)	19 (4)					
6: Epoxi-15	4 (3)	5 (3)					
7: Manc-6,25	11(4)	11(4)					
8: Manc-25	13 (5)	14 (5)					
9: Prochl-8,75	19 (6)	20 (6)					
10: Prochlz-35	10 (3)	11 (3)					
11: Tebu-12,5	25 (4)	25 (4)					
12: Tebu-50	11 (4)	11 (4)					
13: Procy-12,5	16 (5)	17 (5)					
14: Procy-50	12 (4)	9 (2)	1	1	1	3(2)	NS p=0.07

Table 3.26 Extended mixture study Genital malformations in male pups scored on PD 16 or 22 at sectioning or *in vivo* on PD 50. Values show the number of animals with score 0 (normal), score 1 (slight penile malformation), score 2 (moderate penile malformation), or score 3 (severe penile malformation). Values in parentheses are the number of litters with offspring having the indicated score.

Dose group	No. anim. (litters)	Score 0	Score 1	Score 2	Score 3	Score 1, 2 or 3	Statistics
1: Control	71 (15)	71 (15)					
2: Pestimix-14,58	96 (17)	92 (17)	4 (4)			4 (4)	NS
3: Pestimix-29,17	53 (9)	50 (9)	2 (2)	1 (1)		3 (2)	NS
4: Pestimix-43,75	67 (14)	56 (14)	4 (3)	5 (4)	2 (2)	11 (6)*	p= 0.002
5: Epoxicon-3,75	38 (7)	36 (7)	2 (1)				
6: Epoxicon-15	11 (4)	11 (4)					
7: Manc-6,25	31 (5)	31 (5)					
8: Manc-25	39 (7)	39 (7)					
9: Prochl-8,75	44 (9)	43 (9)	0	1(1)		1 (1)	NS
10: Prochl-35	26 (4)	25 (4)	1 (1)			1 (1)	NS
11: Tebu-12,5	48 (8)	48 (8)					

12: Tebu-50	27 (6)	26 (6)	1(1)			1 (1)	NS
13: Procymi-12,5	38 (7)	38 (7)					
14: Procymi-50	25 (4)	19 (4)	2 (2)	3 (2)	1 (1)	6 (2)*	P= 0.035

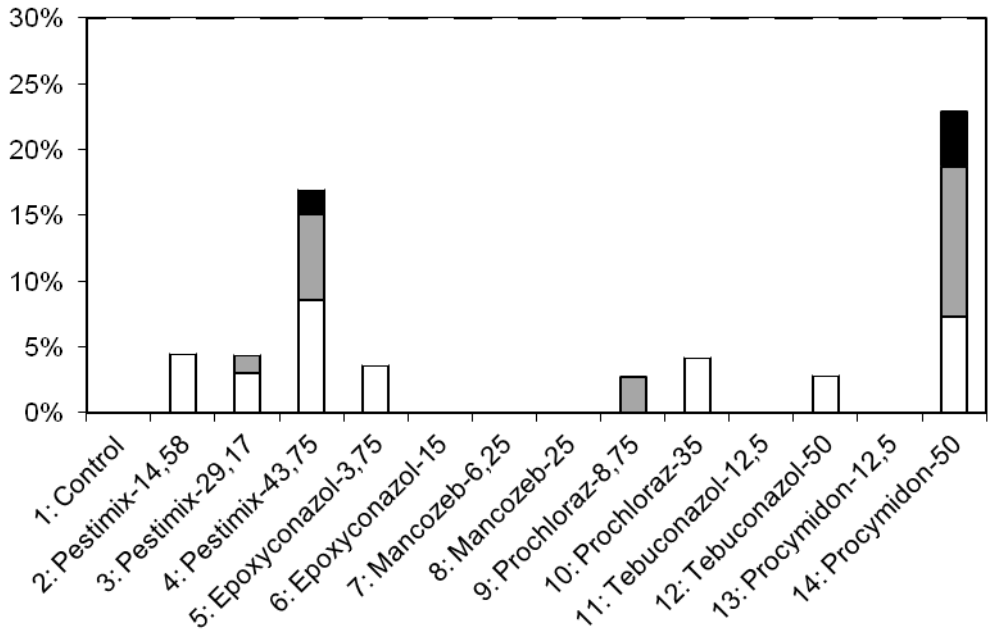


Figure 3.12. Extended mixture study. Genital malformations in male offspring. Results shown are the group mean of the % of the male offspring within each litter given score 1 (white), 2 (gray) or 3 (black). The rest of the animals, i.e. up to 100% total, in each group were given score 0. The incidence of genital malformations is significantly increased in the highest mixture group (group 4) and the high dose of procymidone (group 14)

3.3.4.8 Estrous cyclicity

Cycle lengths of less than 4 days or more than 5 days (e.g. 3 or 7 days), were considered as abnormal and the animals were classified as having normal (regular), short or long cycli, respectively. Animals showing an initial estrous and then becoming acyclic for a minimum of 12 days were considered pseudo pregnant. No exposure related effect on estrous cycle was found in any group when compared to control either when pseudo pregnant animals were in the analysis or left out of the data (fig 3.13).

However, because of the large variation in the control group, the sensitivity for detecting an effect such as an increased number of animals with irregular cycles is limited. Actually, about 87% of the animals in for example group 4 (8 animals total) would have to cycle irregularly to be significantly different from the control group.

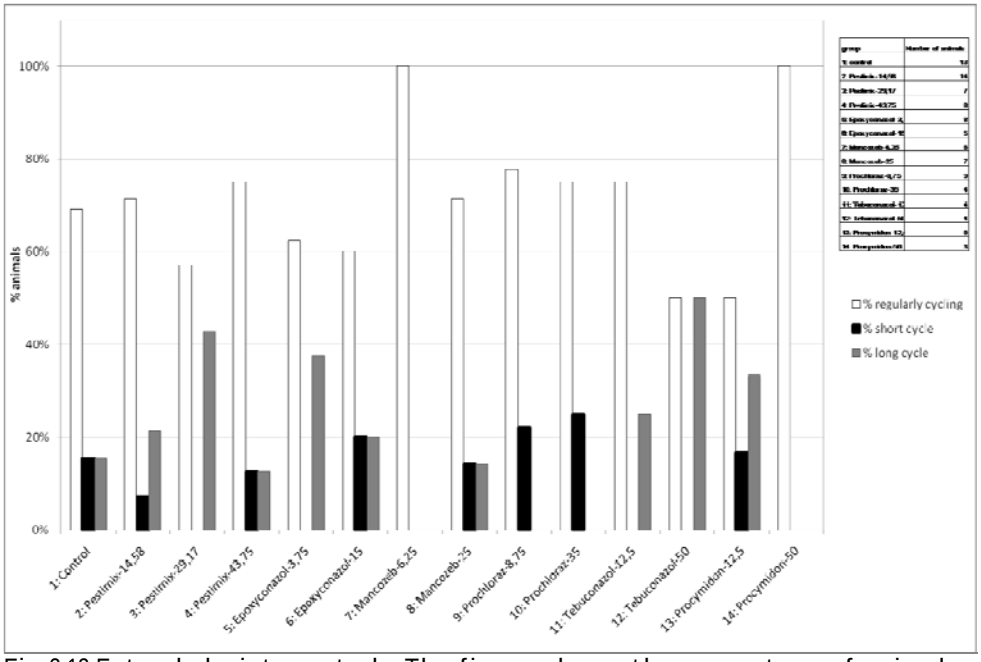


Fig 3.13 Extended mixture study. The figure shows the percentage of animals classified as having normal, short or long estrous cycli in the various groups. In this figure the pseudo pregnant animals have been omitted from the data. Number of animals in each group is shown to the right. No statistical significant effects of exposure were seen on estrous cyclicity.

3.3.4.9 Semen quality

The sperm count in the adult males was significantly lowered in the high dose pestimix group, compared to controls (Figure 3.14). The sperm count results are shown as number of sperm per gram cauda epididymis, and the observed reduction was not due to effects on the weight of the epididymis, as this was unaffected by the exposure (data not shown) . Sperm motility parameters were investigated in all 14 dose groups, but neither % motile sperm nor % progressive sperm were significantly affected by pesticide exposure (data not shown).

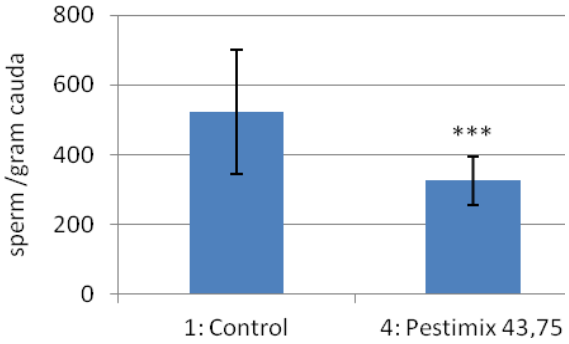


Fig 3.14 Extended mixture study. Number of sperm per gram cauda epididymis in the adult males from the control group, and the group dosed with the highest dose of the pesti-mix during gestation and lactation. Shown are group means ± standard deviations. p-value is ***<0.001

3.3.4.10 Activity

The studies of activity level in adult offspring showed an overall statistically significant effect of sex and group (fig. 3.14). The sexual dimorphic

difference was as expected with females showing higher activity levels than males ($p<0.0001$).

The effect of pesticide exposure was statistically significant in females ($p=0.04$), but not in males. Further analysis showed significantly higher activity level in the females exposed to mancozeb (high dose)($p=0.007$), prochloraz (low dose) ($p=0.029$) and tebuconazole (low dose) ($p=0.029$), whereas no statistically significant effects were seen in the females exposed to the pesticide mixture or to procymidone. As the effect of prochloraz and tebuconazole was seen only at the low dose and not at the 4 times higher dose these changes may be random findings. This is also supported by the lack of effect in the mixture group 3 as this group was exposed to both prochloraz and tebuconazole at the same dose levels. The effect of mancozeb at the high dose could be a real finding, but other studies in our laboratory using a larger group sixe have not found this (Axelstad et al., 2011). Consequently, this finding may also be a false-positive result due to the relatively low number of offspring in the mancozeb group.

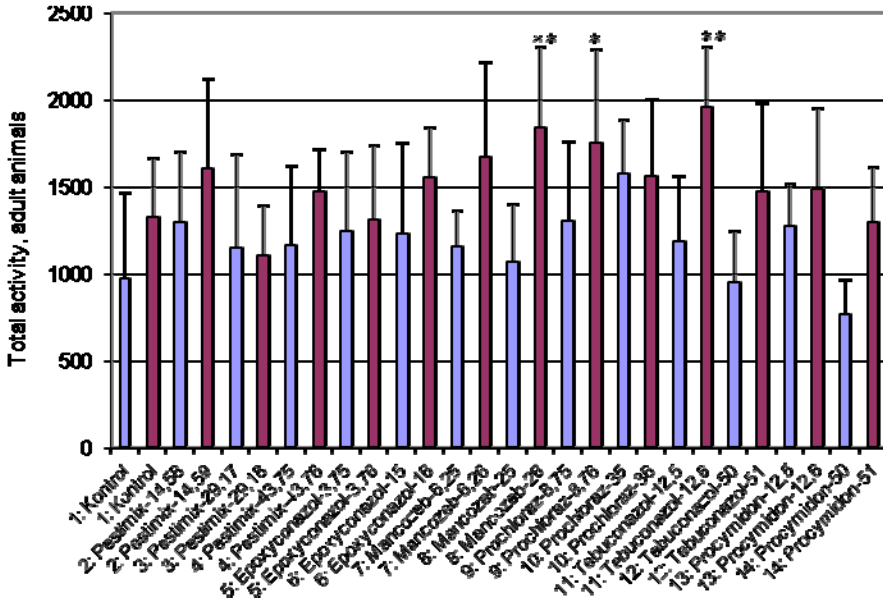


Figure 3.14 Extended mixture study. Total activity levels in adult male (blue columns) and female (red columns) offspring, shown as group means ± SD. A statistically significant difference between sexes within the same dose group was present in groups 1, 2, 4, 7, 8, 9, 11, 12 and14 (not marked on the graph). No exposure related effects were seen in the activity levels of the adult male offspring, while statistically significant differences between exposed and control females are shown with asterisks. p-values are *<0.05, ** < 0.01

3.3.4.11 Learning and memory

A statistically significant sex difference in Morris maze performance was, as expected, observed on the last four days of the learning period (days 4-7), with females spending more time in the water and swimming further before reaching the platform than males (data not shown). The results from the two sexes were therefore analyzed separately, when testing for effects of developmental pesticide exposure on Morris maze performance. No statistically significant effects of pesticide exposure on swim speed were observed in either males or females.

In females no statistically significant effects of exposure to the mixture of pesticides or the single compounds was seen on swim length (fig 3.15) or on latencies to reach the platform (data not shown). The data were analyzed both with all 14 groups, and because of the small group size in groups 5-14, data were also analyzed with only nine groups (pairing the two doses of each pesticide in a single group). No exposure effects were seen in any of these analyses.

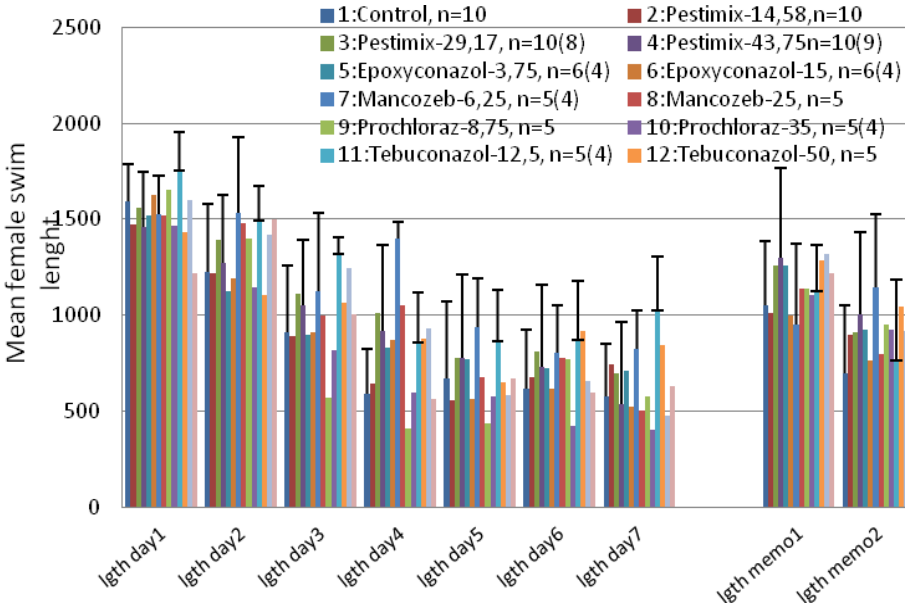


Figure 3.15 Extended mixture study. Mean female swim length in Morris water maze on the 9 days of testing, shown as group means ± SD. No statistically significant differences were observed between exposed and control offspring. The legend shows the number of tested animals in each group, and in parenthesis the number of litter they represent.

All statistics on Morris maze data in males was done on litter means. When data from all 14 groups were analyzed together, total swim lengths during the learning period (days 1-7) were significantly increased in the highest mixture dose (group 4, p=0.0322) and in the low dose tebuconazol animals (group 11, p=0.0004), compared to controls (fig 3.16). The effect of pesticide exposure on male swim length was primarily observed at the end of the learning period (days 6 and 7). When data from each of the 9 days was analyzed separately a tendency towards higher values was seen in the low tebuconazole group (group 11) on day 6 (p=0.0625) and a statistically significant effect was seen on day 7 (p=0.048), whereas the results in the highest mixture group (group 4) were not significantly different from controls on any of the single days (fig. 3.17).

When the data were analyzed as coming from 9 groups instead of 14, no statistically significant effects on swim length were seen, and this was also the case when data from the first 4 groups were analyzed separately - as swim lengths on day 6, 7 and totally had p-values of 11.1%, 6.9% and 10.99% respectively. The higher mean values in groups 4 and 11 were primarily caused by a few animals that still had very high latency and swim length values at the end of the learning period, while all males in the control group at this point had efficiently learned to find the platform.

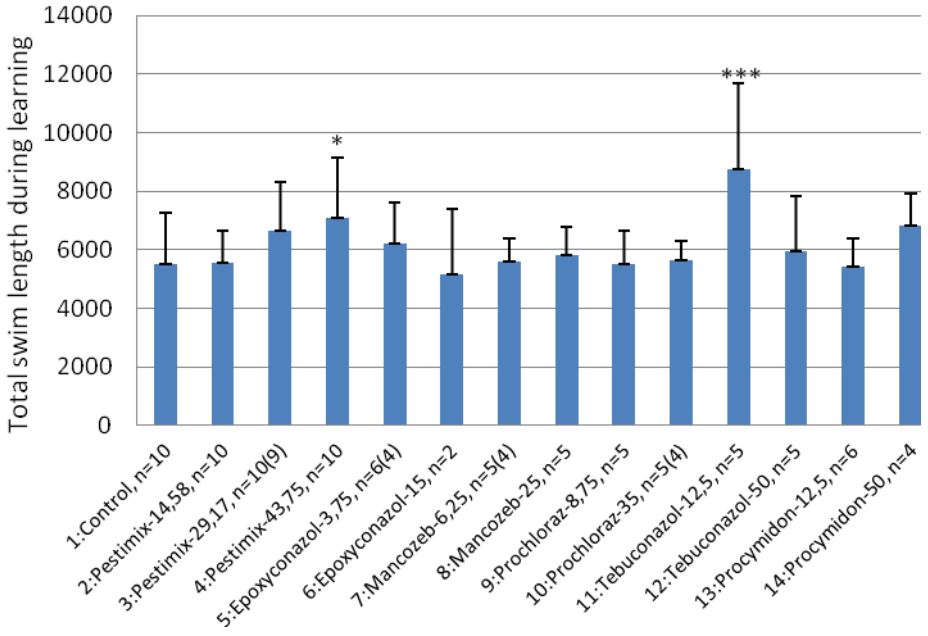


Figure 3.16 Extended mixture study. Total swim length in males during the learning period (days 1-7), shown as group means ± SD. Statistically significant differences between exposed and control offspring are shown with asterisks. p-values are *< 0.05, *** < 0.001

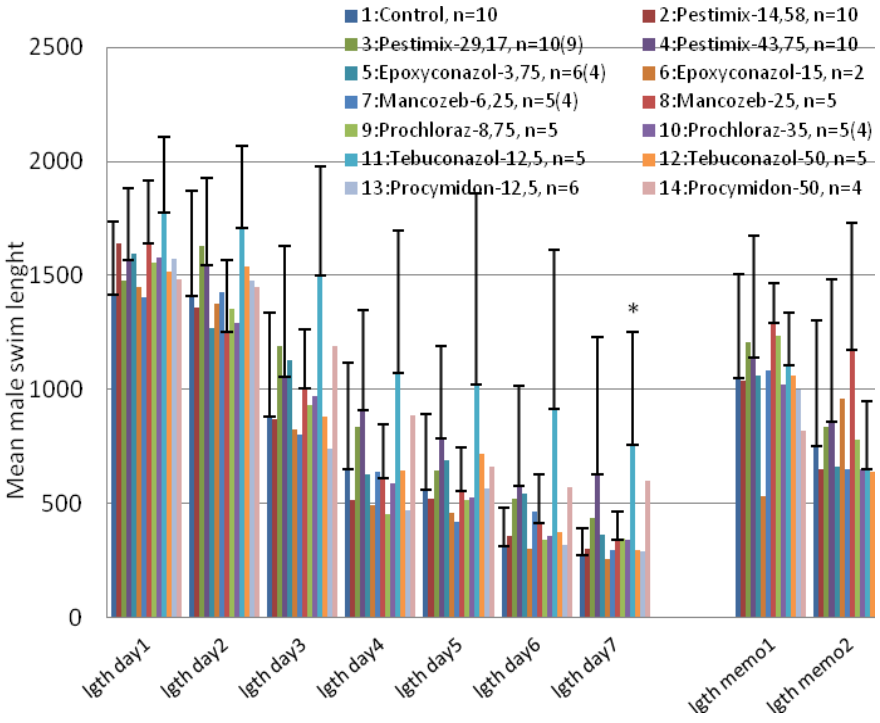


Figure 3.17 Extended mixture study. Mean male swim length in Morris water maze on the 9 days of testing, shown as group means ± SD. The legend shows the number of tested animals in each group, and in parenthesis the number of litter they represent. Statistically significant differences between exposed and control offspring are shown with asterisks. p-values are *< 0.05

The latency data resembled those for swim length, but here an effect was also observed in the high dose procymidone group (fig. 3.18). When data from all 14 groups were analyzed together, total swim latencies during the first 7 days of testing were significantly increased in the highest mixture dose (group 4, p=0.0194), in the low dose tebuconazol animals (group 11, p=0.0001), and in the high dose procymidone animals (group 14, p=0.018) compared to controls (data not shown). The effect of pesticide exposure on male swim latencies was also primarily observed at the end of the learning period (days 6 and 7). When data from each of the 9 days were analyzed separately a statistically significant increase in latency was seen in highest mixture group on day 7 (p=0.0105), in the low dose tebuconazole group on day 6 (p=0.0214) and day 7 (p=0.0021), and in the high dose procymidone group on day 7 (p=0.0036). When the data was analyzed as coming from 9 groups instead of 14, no statistically significant effects on swim latency was seen. When the data from the first 4 groups, i.e. control and the 3 mixture groups, were analyzed separately a significantly higher latency to reach the platform was seen in the highest mixture group on day 7 (p=0.049) and a tendency was seen for the total learning period (p=0.0708).

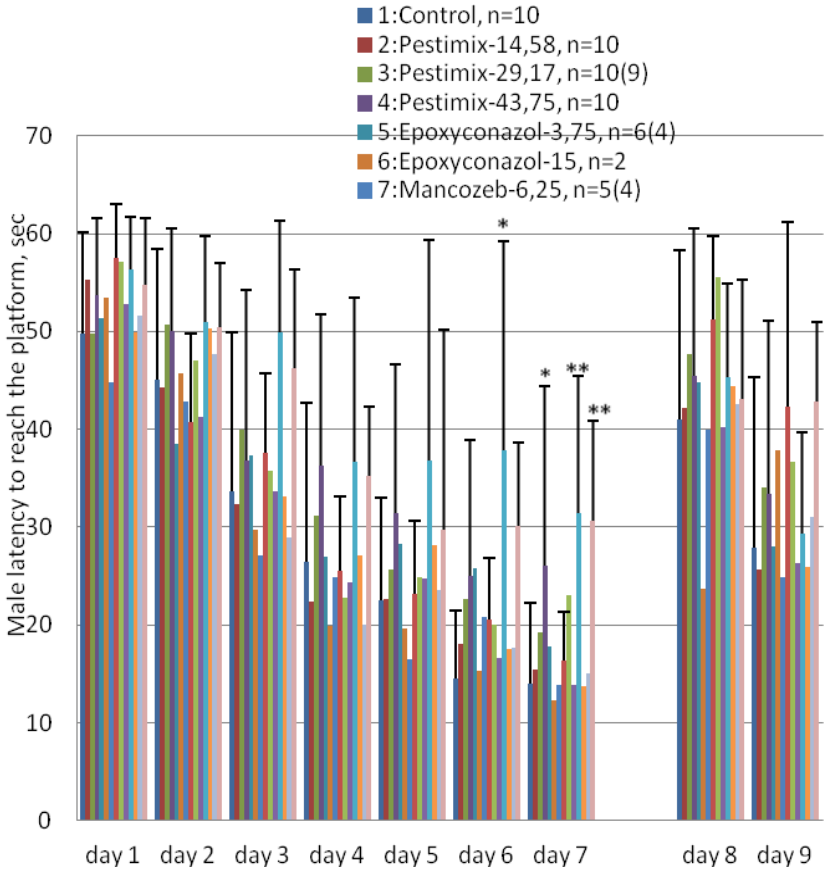


Figure 3.18 Extended mixture study. Mean male latency in Morris water maze on the 9 days of testing, shown as group means ± SD. The legend shows the number of tested animals in each group, and in parenthesis the number of litters they represent if more than one per litter was tested.Statistically significant differences between exposed and control offspring are shown with asterisks. p-values are *< 0.05, **< 0.01

3.3.4.12 Mating behaviour

The results represent data from the assessment of mating behaviour of 87 males (from all groups), as one of the 88 recordings was spoiled. No significant differences in mating behaviour were revealed between the control

and the three pestimix groups. There was a relatively high number of males in each group (n=9-10, from 9-10 litters) and no statistically significant differences for the parameters: “mount”, “intromission” (frequency and latency), or “ejaculation” between control and mixture exposed rats were observed. Moreover, no significant differences in mating behaviour were revealed between the control and the single pesticides groups. In fig 3.19 the mounting frequency is shown.

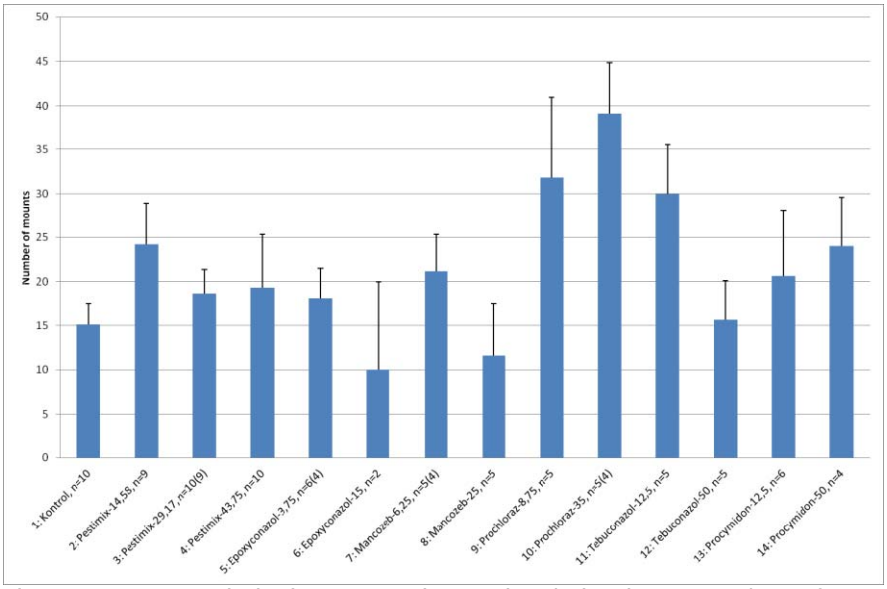


Figure 3.19. Extended mixture study. Mating behaviour, number of mounts during a 20 min. period, shown as group means ± SEM. The number of males is shown as n, while the number in parenthesis is the number of litters.

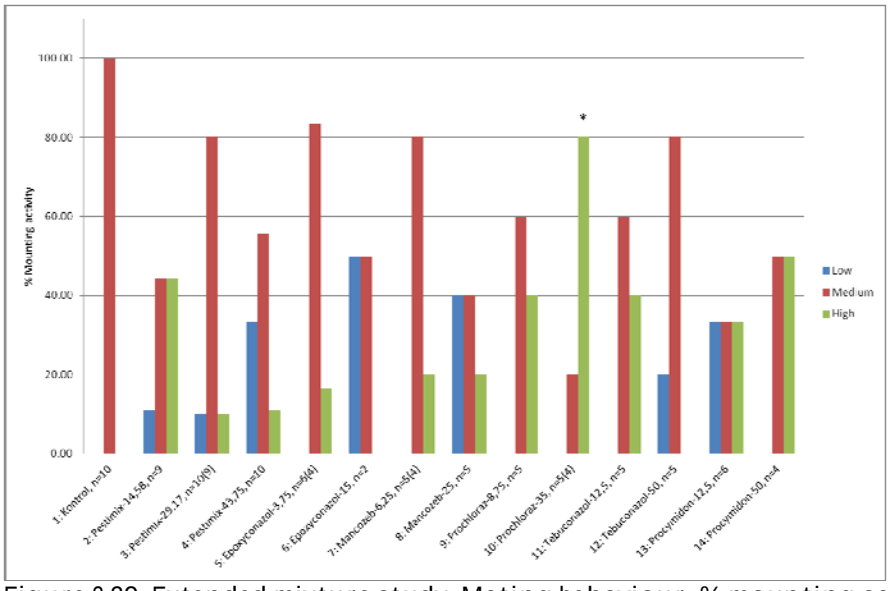


Figure 3.20. Extended mixture study. Mating behaviour, % mounting activity and distribution in 3 groups. Low (number of mounts ≤ 5), medium (number of mounts >5 and <30) or high (number of mounts ≥30), during a 20 min. period, shown as group means (%). The number of males is shown as n, while the number in parenthesis is the number of litters.

When the animals were grouped for general mating activity, as being either low (i.e. number of mounts below or equal to 5), medium (number of mounts

between 5 and 30) or high (number of mounts equal to or above 30), the males from the highest dose of prochloraz showed statistically significantly increased mating activity ($p<0.001$) compared to the controls. In fig 3.20, the distribution in these different groups in relation to mounting frequency is shown. The group size for the single pesticide exposures was however very small in regard to assessment of behavioural results (n=2-6). Consequently, the significantly increased mating activity in the males exposed to the high dose of prochloraz during development can only be considered as suggestive.

3.3.4.13 Hormone levels

There were no statistically significant effects of exposure to the mixture on any of the measured plasma hormone levels in dams or in male or female pups (table 3.27).
Also, there were no statistically significant effects of exposure to the single pesticides on progesterone and testosterone levels in adult offspring.

Table 3.27 - Extended mixture study. Hormone levels in pups and dams. Data represent the effect on hormone levels measured in plasma. N= 3-5 in each group

	Male pups		Female pups		Dams
	PD16	PD50/51	PD16	PD50/51	PD22
Progesterone levels	65±79	159±130	99±21	127±109	132±89
Testosterone levels	137±154	63±65			
Estradiol levels			108±34	128±122	103±47
T4 levels		90±16		109±33	

The shown values are the hormone levels as % of the levels in the control group (= 100 %) for the highest mixture dose-group (43.75 mg/kg/day). However, there was no statistically significant effect seen in any of the mixture dose-groups compared to controls.

3.4 Summary of main findings

Gestation length was significantly increased in the two highest mixture groups and in the group exposed to the highest dose of epoxiconazole, but not in any other groups. As the highest dose of epoxiconazole given alone was 4 times higher than the dose included in the highest mixture group, these results showed combination effect of the pesticides at dose levels where the individual pesticides caused no effects.

Nipple retention in male offspring was significantly higher in all mixture groups, but also at the highest dose of prochloraz, tebuconazole and both doses of procymidone, compared to controls. Since the magnitude of the effect in the highest mixture group was significantly higher that the effect induced by procymidone alone at the dose level included in the highest mixture, the results also indicate mixture effects for this endpoint.

Significant effects on male reproductive organ weight, reduced sperm count and genital malformations were also seen in the highest mixture group, whereas no significant effects were seen for the single compound exposure (not investigated for sperm count). On PD 16, reduced weights of prostate and seminal vesicles and epididymis were seen in the highest mixture dose group. None of the pesticides caused statistically significant effects on these endpoints when given alone. In adult male offspring, reduced weight of reproductive organs was also seen in the highest mixture dose group compared to controls, but not in any of the groups exposed to the pesticides

alone. Therefore, these results show mixture effect at dose levels where the single chemicals did not induce effects.

Increased frequency and severity of genital malformation was also found in the highest mixture group. The percentage of male offspring with genital malformations was markedly higher in the highest mixture group than in any of the groups dosed with the individual pesticides alone at the dose included in the mixture. As such, these results show severe mixture effects at dose levels where the individual pesticides caused no effect when given alone.

In the female offspring, significantly longer AGD index was seen in the two highest mixture groups. A similar effect was seen in animals exposed to both doses of prochloraz and tebuconazol and in the group exposed to the low dose of epoxiconazol but not in animals dosed with a four times higher dose. Therefore, the effect in the mixture groups may or may not be a combination effect.

4 Mixture modelling

4.1 Introduction

The purpose of this work was to investigate if modelling of the expected mixture effects based on data for the single pesticides and using dose-addition or independent action would give a useful estimate compared to the observed in vivo mixture effects for endpoints.

The data for the single pesticides comprised the raw data from our previous studies (see table 2.4 in chapter 2) and the data for both single pesticides and the mixture obtained in the extended mixture study.

Endpoints considered relevant for this modelling were those observed to show combination effects at mixture dose levels where the individual pesticides when given alone caused no or significantly smaller effects. These endpoints include nipple retention in male offspring, gestation length, and anogenital distance in female offspring, some male reproductive organ weights and learning ability. The modelling was, however, not done for the two latter endpoints due to lack of sufficient data for the single pesticides.

4.2 Methods

Data were normalized with regards to the control groups of each year (experiment) such that year did not have to be included in the model as an extra factor, since there were few doses per year. It is desirable to include a higher number of doses to make a better fit of the regression model, and this is only possible when data from experiments of all years ae included. The effect of year was tested using a hypothesis test with null hypothesis no difference in means over years. Either t.test() in R was used for the tests, or lme() in R was used for tests. The latter is sued when sex likewise is included as a factor.

The endpoint nipple retention (NR) was scaled using the mean of the female controls for each year (approximately 12 to 12.5). Endpoints with values larger than this maximum were thresholded to the maximum. This means that the end point was scaled to lie in the interval [0, 1]. It can be argued that NR is a discrete value, and that the transformation into a continuous variable is not optimal. The transformation can however be considered as the proportions of nipples developed in male pups. After scaling according to the female controls the controls were not included in the analyses, but they are plotted in the figures as reference points.

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

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

For NR in males and AGDI in females, the same model and fitting method was used as there for these endpoints are several observations per litter. First, a binomial logit curve was fitted to the observations for each of the pesticides (epoxiconazole, mancozeb, prochloraz, tebuconazole, and procymidone). Generalized estimating equations (GEEs) were used to perform the fit (Liang & Zeger, 1986; Yan & Fine, 2004; Zeger & Liang 1986). This method is particularly good when accounting for correlations within subject (here litter), but independence across litters, i.e. the model is based on pup level but taking the litter effect into account. The variance of the estimates can be incorrect if the correlation structure is not accounted for. In order to keep the number of parameters to a minimum in this model we assume that the correlation is so called "exchangeable", meaning that it is the same for any pair of litters. The model used looks like this:

$$\text{logit}(\boldsymbol{NR}_{\boldsymbol{scaled,il}}) = \boldsymbol{y}_{\boldsymbol{il}} = \alpha + \beta \cdot \boldsymbol{dose}_{\boldsymbol{il}},\ \boldsymbol{i=1,\ldots N,\ l=1,\ldots L_i}$$

where α and β are the parameters to be estimated, $\boldsymbol{N}$ is the number of litters, and $\boldsymbol{L_i}$ is the number of pups in the $\boldsymbol{i}^{\text{th}}$ litter. The effect of dose is assumed fixed. The effect from litter is incorporated in the correlation structure of the model fit, and by using the exchangeable correlation structure it is assumed that correlations between pups in a litter is the same for each litter. This is done based on the fitted (wrong) independence model above, where we estimate the Pearson residuals as

$$r_{il} = \frac{y_{il} - \hat{\mu}_{il}}{\sqrt{\hat{\mu}_{il}(1-\hat{\mu}_{il})}}, i = 1,\ldots N, l = 1,\ldots,L_i$$

where $\boldsymbol{i}$ is the $\boldsymbol{i}^{\text{th}}$ litter, and $\boldsymbol{L_i}$ is the number of pups in the litter. We use the Pearson residuals to estimate the correlation matrix of correlations between pups in the same litter as

$$corr(y_{il}, y_{il'}) = \hat{\omega} = \phi \sum_{i=1}^{N} \sum_{l>l'} \hat{r}_{il} \hat{r}_{il'} \Big/ \left(\sum_{i=1} 1/2 L_i (L_i - 1) - p \right),$$

where $\boldsymbol{p}$ is the number of parameters, ω is also called the correlation parameter, and Φ is the scale parameter which can be estimated by

$$\hat{\phi}^{-1} = \sum_{i=1}^{N} \sum_{l=1}^{L_i} \hat{r}_{il}^{\,2} / (\sum_i L_i - p).$$

The models were fitted using the function `gee()` in the R package gee from CRAN [ref: Vincent J Carey. Ported to R by Thomas Lumley (versions 3.13, 4.4) and Brian Ripley (version 4.13). (2009). gee: Generalized Estimation Equation solver. R package version 4.13-14. http://CRAN.R-project.org/package=gee].

For GL, a generalized linear model (GLM) was fitted to all observations for the five pesticides and the pesticide mixture. As GL is measured on mother level, there is only an observation per litter, and not on pup level, therefore we now use a GLM rather than a GEE. The model used is:

$$\text{logit}(\boldsymbol{NR}_{scaled,i}) = \alpha + \beta \cdot \boldsymbol{dose}_i \text{ , } \boldsymbol{i=1,\ldots,N}$$

where $\boldsymbol{i}$ is the $\boldsymbol{i}^{th}$ mother, and $\boldsymbol{N}$ is the number of mothers, α and β are the parameters to be estimated, and the residual ε is assumed normal distributed. Here, there is no correlation structure for litters as the GL is measured for the mothers. The `glm()` function in R was used for estimation and prediction.

The same model was fitted to the same endpoints for both single pesticides and the mixture.

Two models for prediction were considered. First, the dose-additivity predictions were estimated. These are defined by the effect dose of the pesticide mixture:

$$\boldsymbol{EDx}_{mixture} = (\boldsymbol{p}_1/\boldsymbol{EDx}_1 + \boldsymbol{p}_2/\boldsymbol{EDx}_2 + \boldsymbol{p}_3/\boldsymbol{EDx}_3 + \boldsymbol{p}_4/\boldsymbol{EDx}_4 + \boldsymbol{p}_5/\boldsymbol{EDx}_5)^{-1}$$

for the effect doses of the five pesticides in question, and where $\boldsymbol{p}_i$ is the proportion of pesticide $\boldsymbol{i}$ in the mixture. The parametric delta method (closely related to parametric bootstrapping) was performed to estimate the $\boldsymbol{EDx}_{mixture}$ from the fitted models and their variance estimates (Efron 1982). The distributions of the parameter estimates were sampled using the estimated variances of the parameters α and β. Random normal samples were used to sample new parameters and thereby obtain predictions using a Monte Carlo sampling strategy assuming a normal distribution of the parameters α and β. 1000 samples were drawn for each parameter, and a dense grid of the doses was used to estimate the effect doses.

Secondly, the independent actions model (Bliss 1939):

$$\boldsymbol{Ex}_{mixture} = \boldsymbol{1-(1-Ex}_1)\cdot(1-\boldsymbol{Ex}_2)\cdot(1-\boldsymbol{Ex}_3)\cdot(1-\boldsymbol{Ex}_4)\cdot(1-\boldsymbol{Ex}_5)$$

was likewise estimated using such a Monte Carlo sampling. Here, $\boldsymbol{Ex}_i$ denotes the fractional effect (in %) of the $\boldsymbol{i}^{th}$ pesticide, where we have $\boldsymbol{x}_i = \boldsymbol{x}_{mixture} \cdot \boldsymbol{p}_i$. Note, that the independent actions formula is given for the effect whereas the dose-additivity model is given in terms of the effect dose.

The same analyses were performed with winsorization, as described in Scholze et al. (2001). This means that the fitting is performed iteratively with observations which falls outside 3 times the standard deviation of the mean prediction are adjusted to the value of 3 times the s.d. and then a new fit is performed etc.

4.3 Results

4.3.1 Nipple retention in male offspring

Tests showed that there was no statistically significant difference in NR over the years (for the different experiments), and thus in the further analysis it was assumed that there was no difference and all experiments were included as one.

Boxplots do not show any strong effects from years, but still some significance was seen for tebuconazole and controls from same year (there is a

convergence problem when year is a fixed effect for tebuconazole and control year 04 and 05). Otherwise, no effect was seen on NR for year.

Table 4.1: Tests for null-hypothesis that there is no difference in NR for years. Sex is included as a fixed effect, and we group on litter.

Pesticide	Dose (years)	Statistically significant difference (year random)	Statistically significant difference (year fixed)
Epoxiconazole	15 (05, 09)	No (28%)	No (24%)
Tebuconazole	50 (05, 09)	Yes (0%)	-
Procymidone	50 (04, 09)	No (46%)	No (39%)
Pestimix	43.75 (09-02, 09-07)	No (69%)	No (72%)
Control	05, 09	-	Yes (0%)
Control	04, 09	-	No (26%)
Control	09-02, 09-07	No (98%)	No (100%)

09-02 and 09-07 are study numbers of the two studies performed in 2009, the 2nd range-fining and the extended mixture study.

In figures 4.1-4.5 the results for the single chemicals are shown with the estimated model and +/- 2 standard errors Note, that there was not observed any statistically significant effect on NR from mancozeb and the effect of mancozeb was therefore set to zero in the predictions of for the mixture effects. Furthermore, due to the tested doses and limitations of the experiments there are high variances on the predictions, in particular for high doses of prochloraz, tebuconazole, and epoxiconazole.

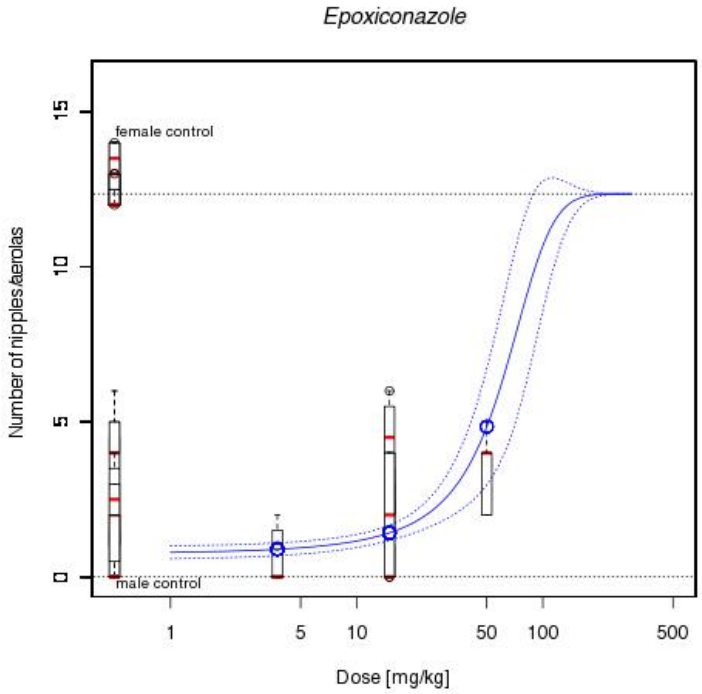


Figure 4.1: GEE fit of epoxiconazole, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means. Note, that +2 times the standard deviation goes beyond the maximum number of NR, this is an artefact in the plot, and can be interpreted as the maximum.

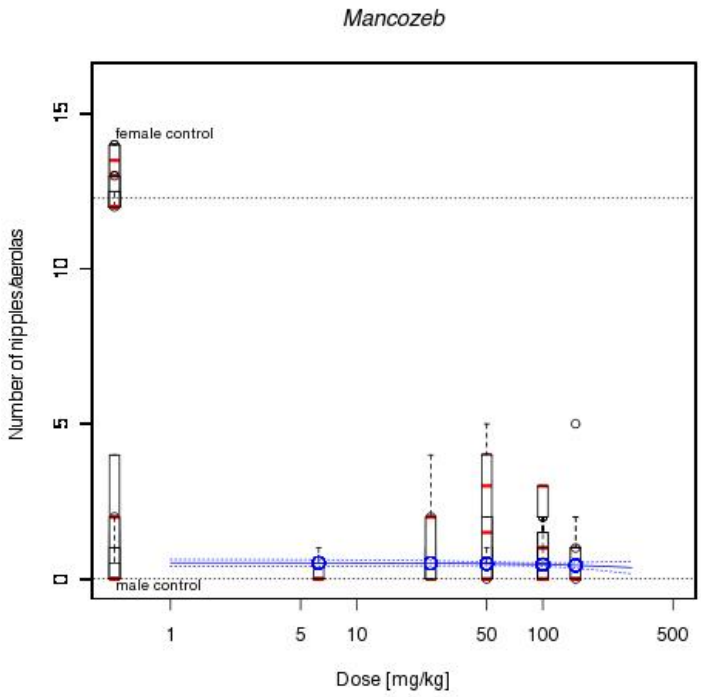


Figure 4.2: GEE fit of mancozeb, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means.

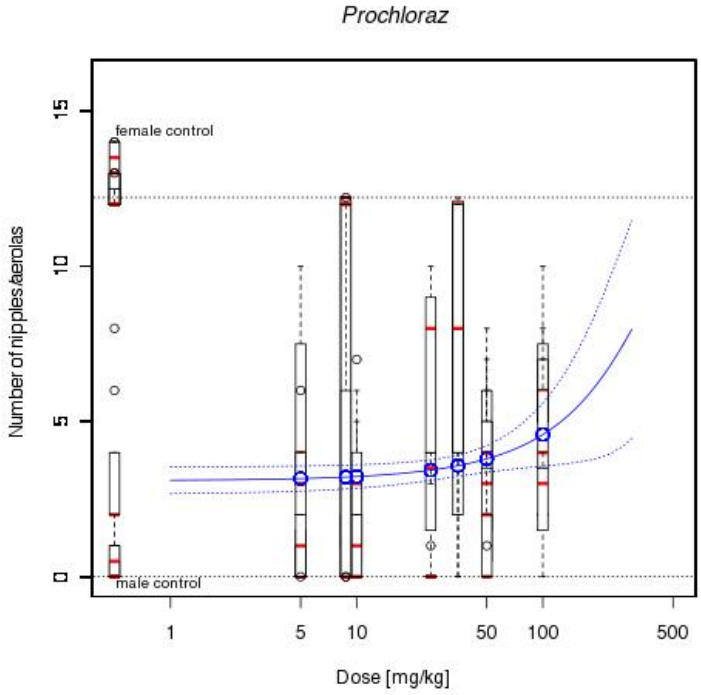


Figure 4.3: GEE fit of prochloraz, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means.

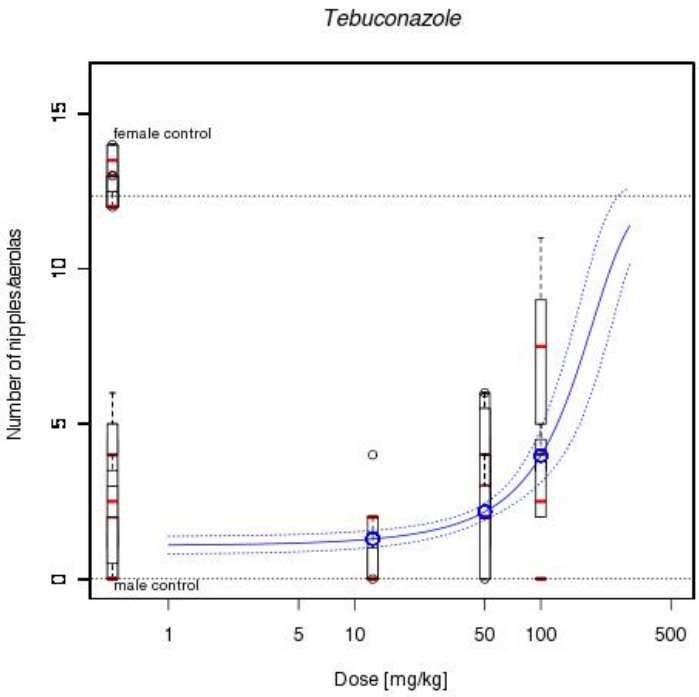


Figure 4.4: GEE fit of tebuconazole, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means. Note, that +2 times the standard deviation goes beyond the maximum number of NR, this is an artefact in the plot, and can be interpreted as the maximum.

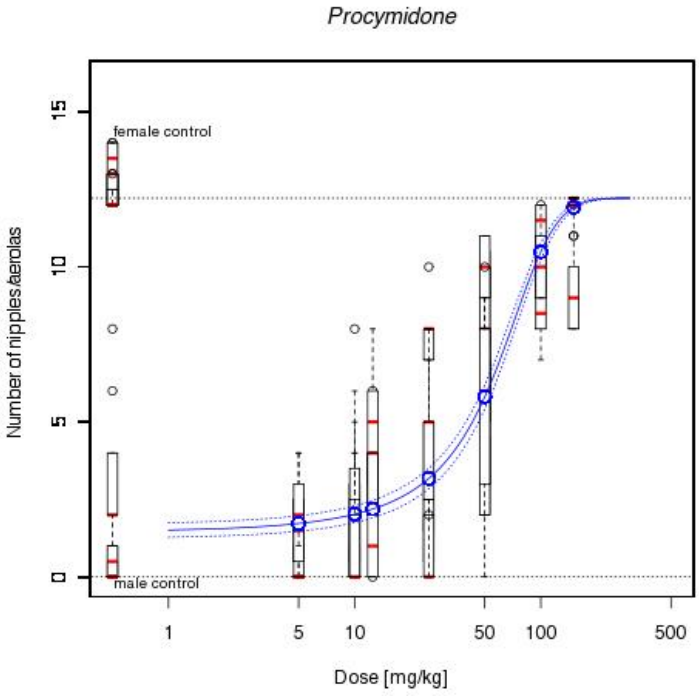


Figure 4.5: GEE fit of procymidone, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means.

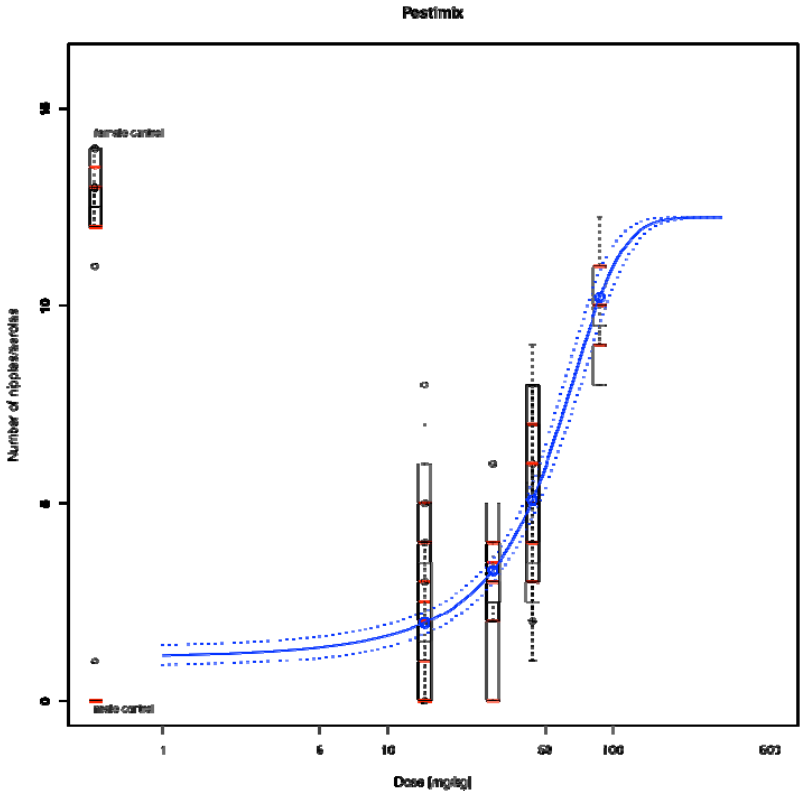


Figure 4.6A: GEE fit of pestimix, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means.

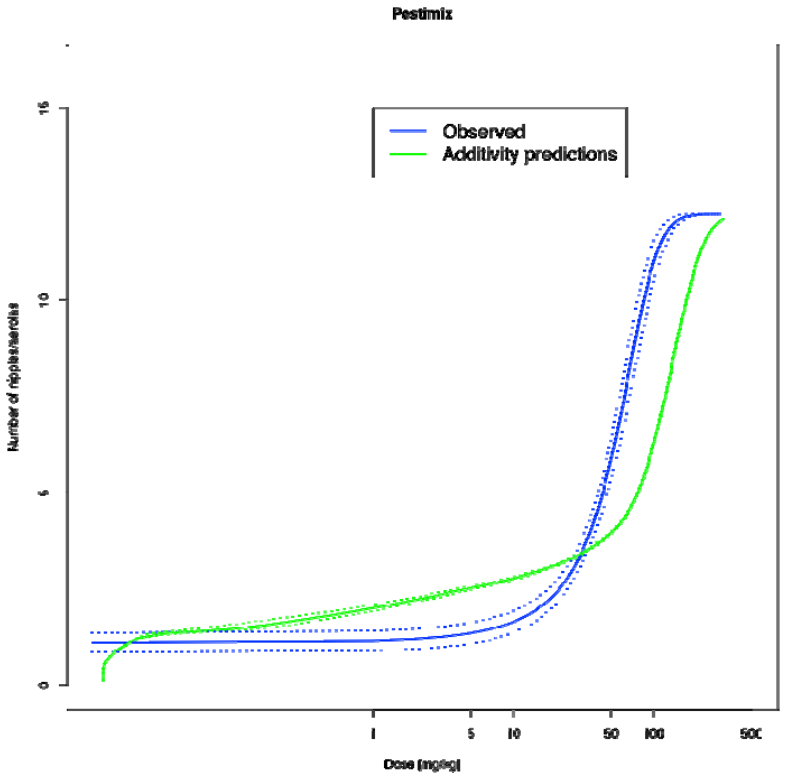


Figure 4.6B: The observed and predicted does effects of NR. The dose-additivity predictions of the effect doses are in green and the observed curve in blue. In dotted lines +/-2 standard errors are illustrated.

The fitted model (in blue) and the dose-additivity predictions (in green) are illustrated in figure 4.6. Furthermore, the 95% confidence intervals and the estimates of the observed, the dose-additivity predictions, and the independent actions predictions are summarized in table 4.2 for the 10%, 25%, 50%, 75%, and 90% effect doses. The results show that at high doses there is no overlap in the confidence intervals between the observed dose effects and those predicted by dose-additivity. In fact, the dose-additivity formula underestimates the effects for the high doses of the multi-component mixture. However, for the low doses where the effects are small the confidence intervals overlap and there are no apparent statistical differences between the observed dose effects and the dose-additivity predictions. The independent actions predictions are seen to likewise underestimate the effects for high doses, and overestimate the effect for the low doses. Furthermore, the independent actions model give poorer estimates in relation to the observed effects in comparison to the the dose-additivity model.

Table 4.2: Predictions of mixture response doses and their 95% confidence intervals (CI).

Relative NR	10%	25%	50%	75%	90%
NR (rounded)	1	3	6	9	11
Mean dose (observed)	2.3	27.0	51.5	76.0	100.5
95% CI (observed)	[0.01-4.9]	[25.5-28.5]	[49.5-53.5]	[72.5-79.5]	[95.0-105.5]
Mean dose (additivity)	0.021	18.85	98.39	153.53	206.45
95% CI (additivity)	[0.020-0.022]	[17.6-20.1]	[97.3-99.5]	[151.9-155.1]	[204.3-208.5]
Mean dose (independent)	0.01	0.01	41.5	184.0	289.0
95% CI (independent)	[0.01-0.01]	[0.01-0.01]	[39.8-43.1]	[182.5-185.5]	[287.4-290.6]

In table 4.3 the effect doses of the pesticides are summarized.

Table 4.3: Effect doses of epoxiconazole, prochloraz, tebuconazole, and procymidone. Mean doses when using Monte Carlo sampling.

Relative NR	10%	25%	50%	75%	90%
Epoxiconazole	12.49	37.59	62.87	88.14	113.41
Procholoraz	14.92	30.39	233.79	421.94	565.69
Tebuconazole	13.76	79.70	149.88	220.08	290.27
Procymidone	0.7713	24.06	53.27	82.47	111.67

Using winsorization, the results were similar, but did differ slightly. In particular, the confidence intervals were much slimmer which reflected that the winsorized data had been adjusted such that “outliers” were corrected. The question is whether these observations should actually be considered outliers, as it was seen from the lines indicating 2 times the standard deviation in the figures of the fits, that it was quite a large number of observations which were adjusted in the winsorization process. Consequently, the data were not used and are not shown.

4.3.2 Gestation length (GL)

We used a two-sided t-test to check if the GL was comparable over years. Table 4.4 summarizes the p-values for the tests on the original observations of GL. We see that there is a statistically significant difference over year for epoxiconazole and tebuconazole.

Table 4.4: Two-sided t-tests of GL over years.

Pesticide	Dose (years)	Statistically significant difference	P-value
Epoxiconazole	15 (05, 09)	Yes	0.013
Tebuconazole	50 (05, 09)	Yes	0.023
Procymidone	50 (04, 09)	No	0.20
Pestimix	43.75 (09-02, 09-07)	No	0.77
Control	05, 09	Yes	0.0028
Control	04, 09	Yes	0.0042
Control	07, 09	Yes	0.0086
Control	09-02, 09-07	No	1.0

Table 4.5 summarizes the p-values for the tests of the transformed GL. We see that after the transformation, there is only one pesticide that has a statistically significant difference over years on a 5% level of significance, namely tebuconazole. Figure 4.7 gives boxplots of the transformed GL for tebuconazole added with a dose of 50 mg/kg for year 2005 and 2009.

Table 4.5: Two-sided t-tests of the GL corrected for the mean of the GL for the controls. T-test in R was used for the tests.

Pesticide	Dosis (years)	Statistically significant difference	P-value
Epoxiconazole	15 (05, 09)	No	0.30
Tebuconazole	50 (05, 09)	Yes	0.030
Procymidone	50 (04, 09)	No	0.080
Pestimix	43.75 (09-02, 09-07)	No	0.77
Control	05, 09	Yes	0.0076
Control	04, 09	Yes	0.0061
Control	07, 09	Yes	0.00043
Control	09-02, 09-07	No	1.0

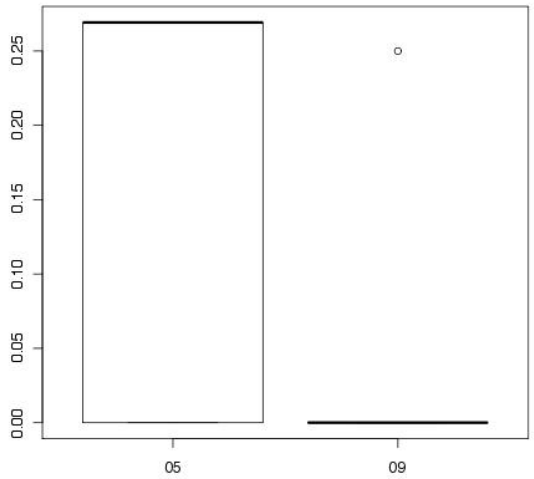
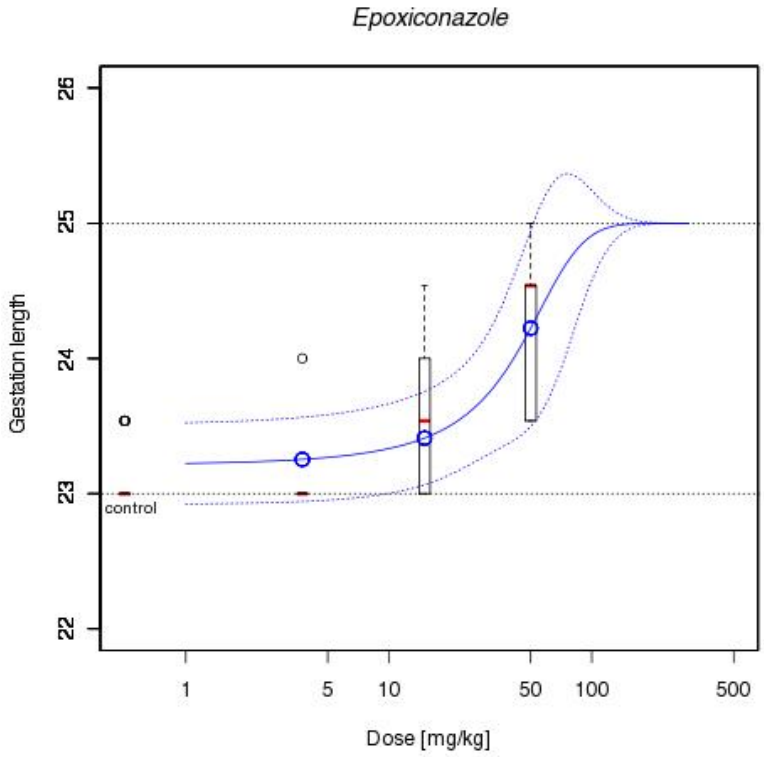
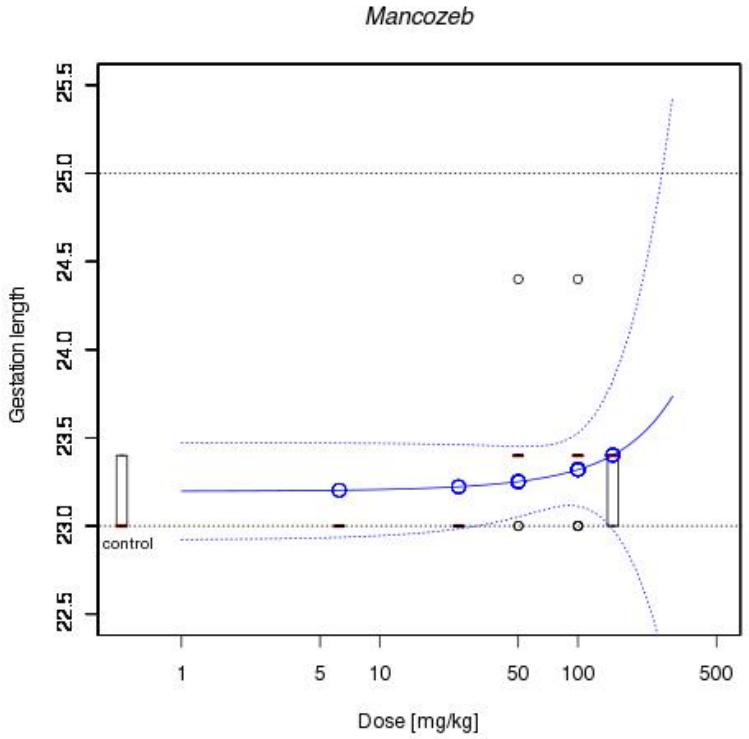
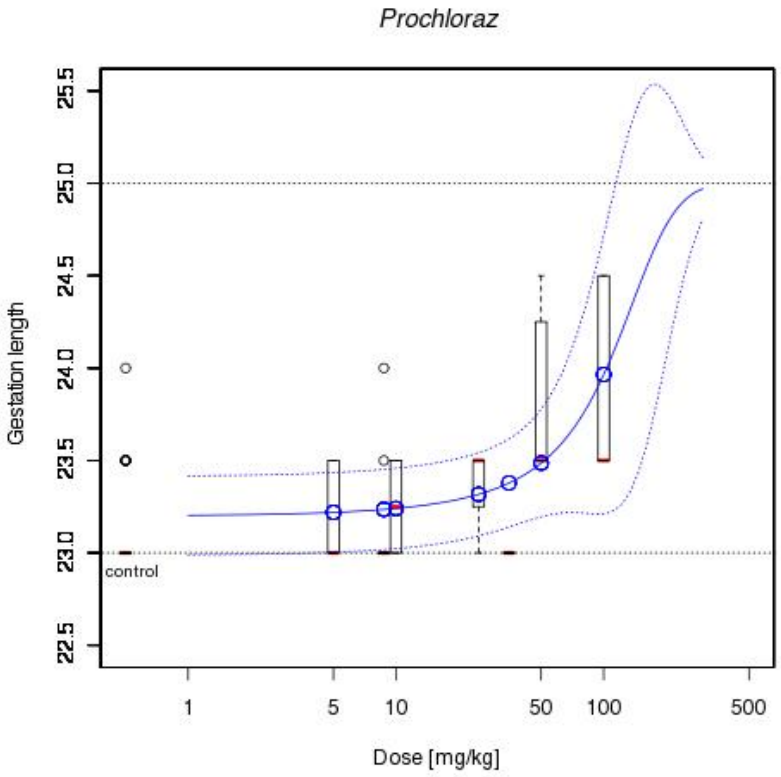


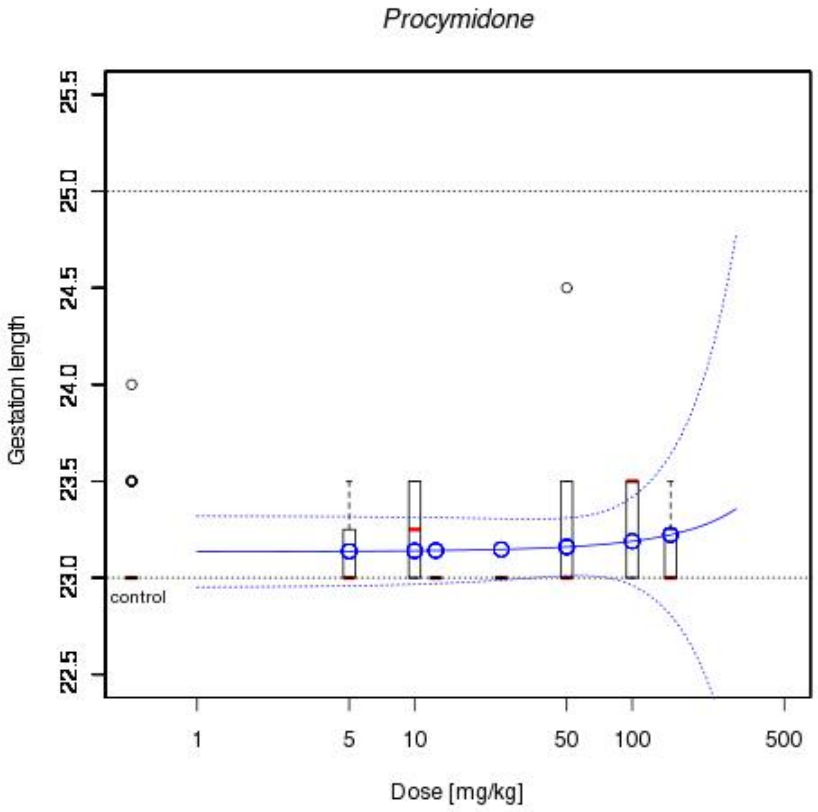
Figure 4.7: Boxplots of the transformed GL for tebuconazole added with a dose of 50 mg/kg and for years 05 and 09.

The results of the GLM fits of GL for the five pesticides are illustrated in figure 4.8.


Epoxiconazole
Gestation length
26
25
24
23
22
control
1
5
10
50
100
500
Dose [mg/kg]


Mancozeb
Gestation length
25.5
25.0
24.5
24.0
23.5
23.0
22.5
control
1
5
10
50
100
500
Dose [mg/kg]


Prochloraz
Gestation length
25.5
25.0
24.5
24.0
23.5
23.0
22.5
control
1
5
10
50
100
500
Dose [mg/kg]


Procymidone
Gestation length
25.5
25.0
24.5
24.0
23.5
23.0
22.5
control
1
5
10
50
100
500
Dose [mg/kg]

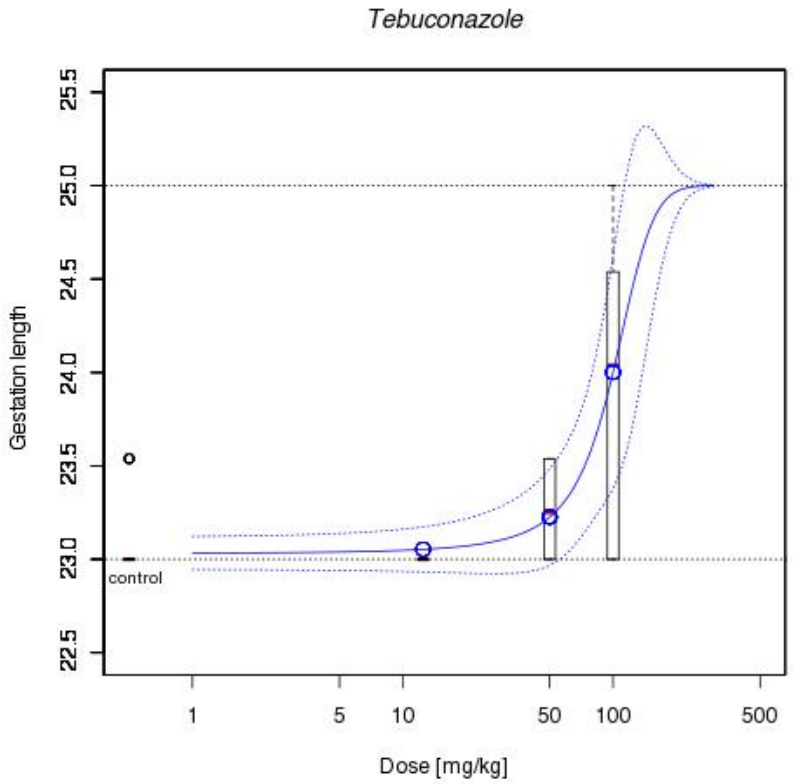


Figure 4.8: GLM fits of GL for added epoxiconazole, mancozeb, prochloraz, procymidone and tebuconazole. +/-2 standard errors are marked with dotted lines and the mean prediction in solid. The boxplots illustrates the observations at the various doses. Note, that +2 times the standard deviation go beyond the maximum GL in some of the plots, this is an artefact in the plot, and can be interpreted as the maximum.

The effect on GL from the mancozeb doses was not statistically significant ($p=0.25$). Likewise, the effect on GL from the procymidone doses was not statistically significant ($p=0.25$). Therefore, mancozeb and procymidone were not included in the predictions for the mixture.

A fit of the observed pesticide mixture was performed similar to the single pesticide observations. In addition, predictions were performed based on the models for the single pesticides and the dose-additivity and independent addition formulas. The results are shown in table 4.6 and figure 4.9. There is a significant difference, and both of the formulas are underestimating the mixture effects at high doses. However, the underestimation is generally smaller for the dose-additivity prediction than the independent action prediction. The dose-addition prediction at low doses, however, gives a good prediction of the observed effects.

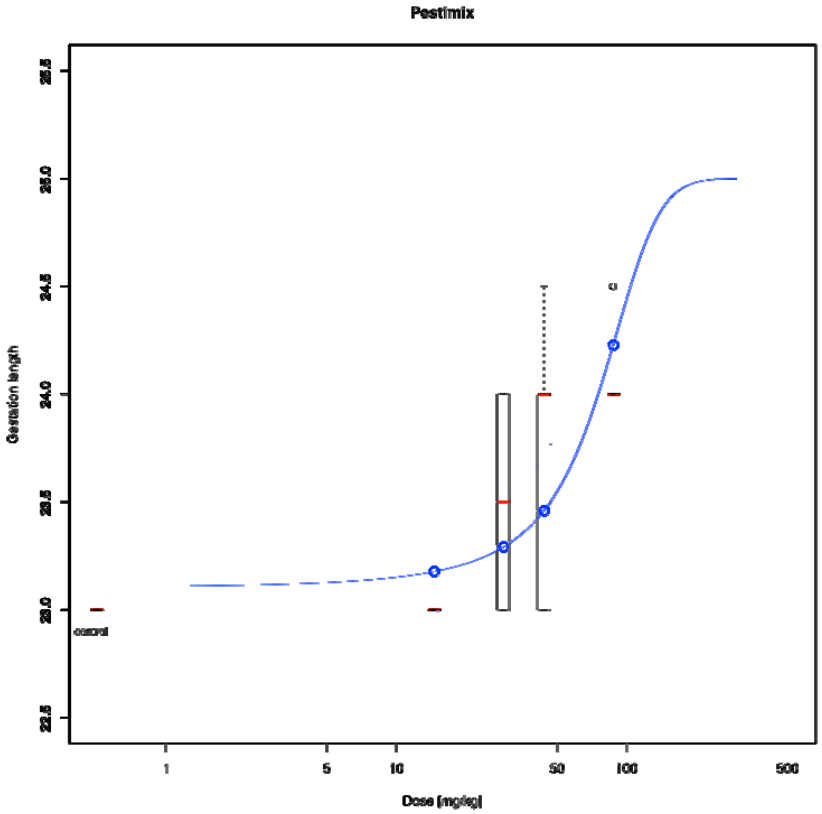


Figure 4.9A: Model and predictions of GL. Note, that +2 times the standard error goes beyond the maximum GL, this is an artefact in the plot, and can be interpreted as the maximum.

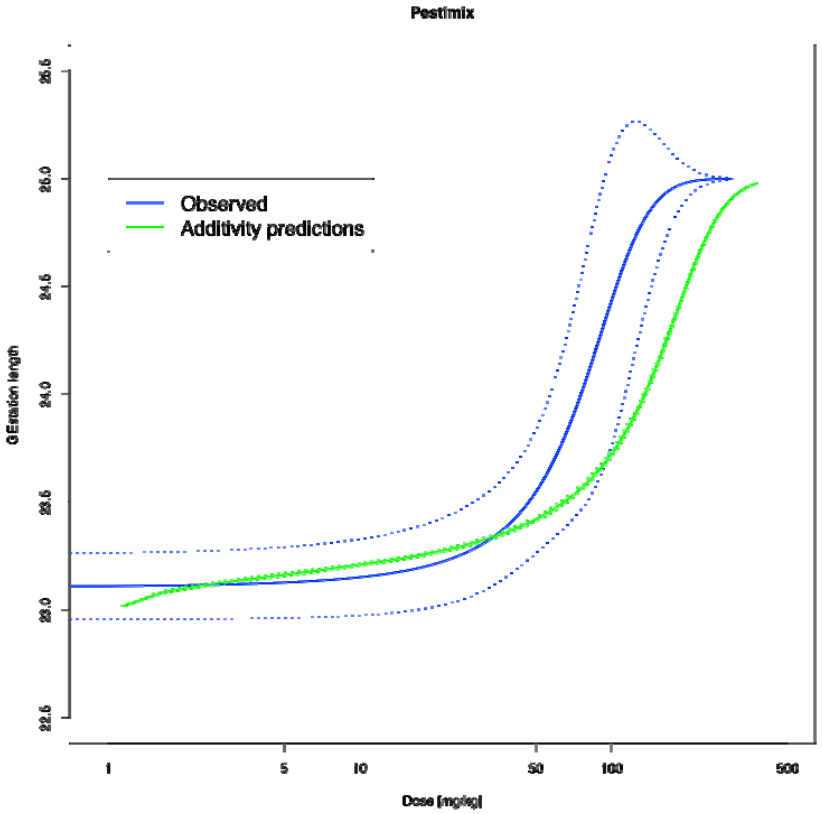


Figure 4.9B: The observed and predicted does effects of GL. The dose-additivity predictions of the effect doses are in green and the observed curve in blue. In dotted lines +/-2 standard errors are illustrated.

Table 4.6: Predictions of mixture response doses and their 95% confidence intervals (CI).

Relative GL	10%	25%	50%	75%	90%
GL (rounded)	23.2	23.5	24.0	24.5	24.8
Mean dose (observed)	18.0	46.5	75.5	104.0	133.0
95% CI (observed)	[3.4-31.0]	[37.5-57.5]	[64.5-93.5]	[85.0-127.0]	[101.5-159.0]
Mean dose (additivity)	9.49	64.0	135.3	197.8	256.2
95% CI (additivity)	[8.50-10.5]	[61.6-66.5]	[132.8-137.9]	[195.1-200.5]	[253.2-259.3]
Mean dose (independent)	0.5	0.5	173.0	316.5	439.5
95% CI (independent)	[0.14-0.86]	[0-4.05]	[167.4-178.6]	[310.5-322.5]	[433.0-446.0]

In table 4.7 the effect doses of the pesticides are summarized.

Table 4.7: Effect doses for individual pesticides. The mean doses of the Monte Carlo sampling.

Relative GL	10%	25%	50%	75%	90%
Epoxiconazole	6.18	23.13	46.18	70.10	93.75
Procholoraz	15.27	64.56	123.1	180.7	237.5
Tebuconazole	48.32	74.39	101.4	128.6	155.8

Using winsorization, the effect doses of prochloraz and tebuconazole were seen to have slightly higher estimates and for epoxiconazole the estimates were slightly lower (data not shown). For the mixture the estimates also got slightly higher when using winsorization. This trend was also seen for the predictions using dose-additivity and independent actions. The overall results when comparing the observed predictions to those using dose-additivity or independent actions were still the same as those without winsorization.

4.3.3 Increased AGDI in female offspring

The following tests are tests with the null-hypothesis that the effect from year is equal to zero. The tests were performed using the linear mixed-effects model (lme() in R) with fixed effect = Sex, Grouped = litter, and year as random and fixed, respectively. The results are summarized in Table 4.8.

Table 4.8: Tests of null-hypothesis that the effect of year on AGDI is equal to zero.

Pesticide	Dosis (years)	Statistically significant difference (year random)	Statistically significant difference (year fixed)
Epoxiconazole	15 (05, 09)	Yes (1.6%)	Yes (1.1%)
Tebuconazole	50 (05, 09)	Yes (0%)	Yes (0%)
Procymidone	50 (04, 09)	Yes (0%)	Yes (0.01%)
Pestimix	43.75 (09-02, 09-07)	Yes (0%)	Yes (0.02%)
Control	04, 09	Yes (0%)	Yes (0%)
Control	05, 09	Yes (0%)	Yes (0%)
Control	09-02, 09-07	No (13%)	No (25%)

There is a rather significant effect of year on AGDI (no matter the year difference). In the boxplots you can visually tell that AGDI increases for the 09-07 experiment compared to the earlier experiments. The same is seen for controls except from 09-02 to 09-07.

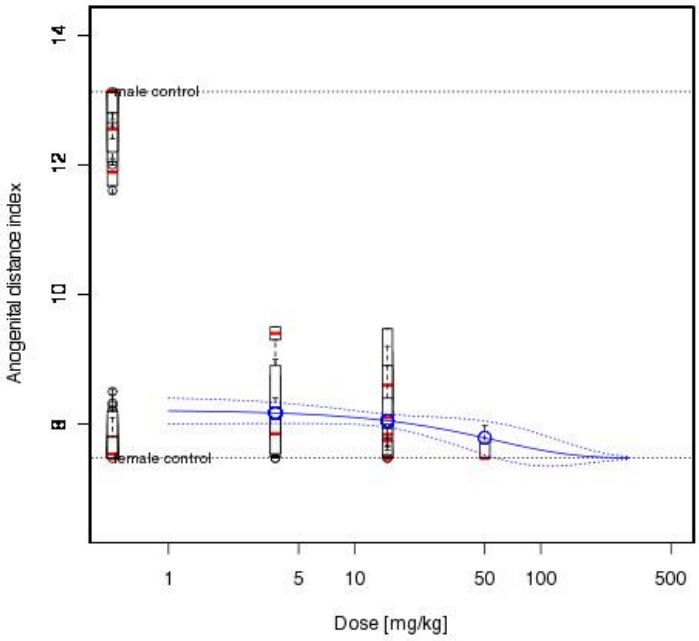
The tests for significance of year were performed again after transformation of the data and the results are seen in table 4.9. Now, the significance of year has diminished and is only statistically significant for tebuconazole and pestimix. We used the transformed AGDI observations in the further analysis where data from all experiments were treated as one.

Table 4.9: Tests of null-hypothesis that the effect of year on transformed AGDI is equal to zero based on transformed data.

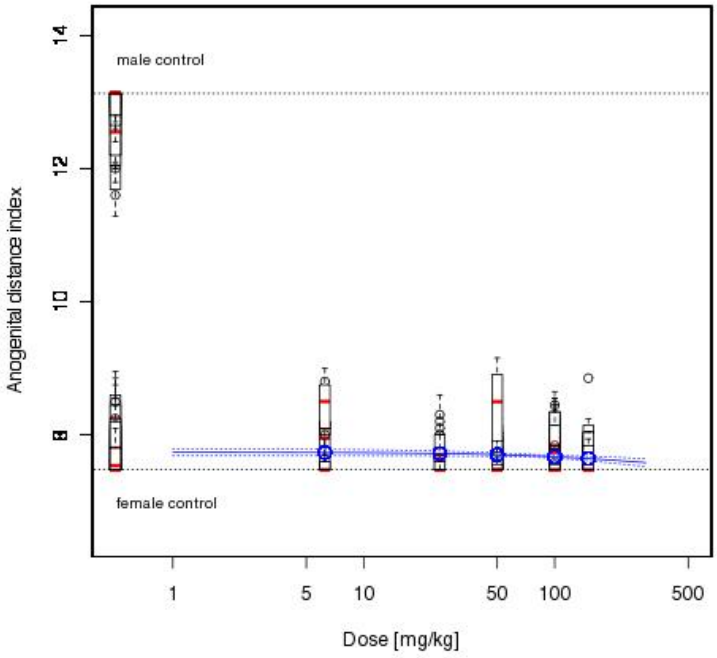
Pesticide	Dosis (years)	Statistically significant difference (year random)	Statistically significant difference (year fixed)
Epoxiconazole	15 (05, 09)	No (74%)	No (72%)
Tebuconazole	50 (05, 09)	Yes (1.2%)	Yes (2.8%)
Procymidone	50 (04, 09)	No (31%)	No (44%)
Pestimix	43.75 (09-02, 09-07)	Yes (0%)	Yes (0.02%)
Control	04, 09	No (96%	No (96%)
Control	05, 09	No (42%)	No (42%)
Control	09-02, 09-07	No(63%)	No (67%)

In the following, we consider the dose effects on AGDI for the female pups only. We use the same model and fitting method (gee) as for NR and the models of the observed individual pesticide effects on AGDI are illustrated in figure 4.10. Only prochloraz can based on this be assumed to have a statistically significant effect on AGDI. The relative few data available for epoxiconazole and tebuconazole suggest that accepting the null-hypothesis of no effect from these pesticides without further examination is arguable.

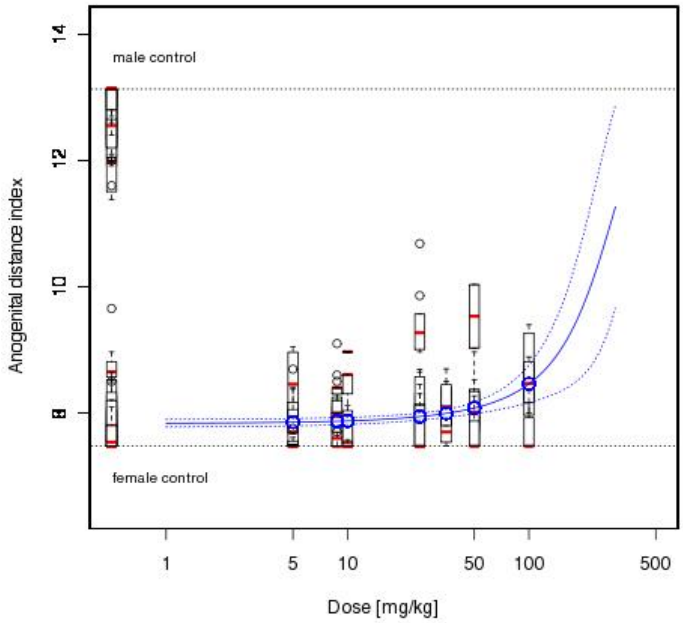
Epoxiconazole



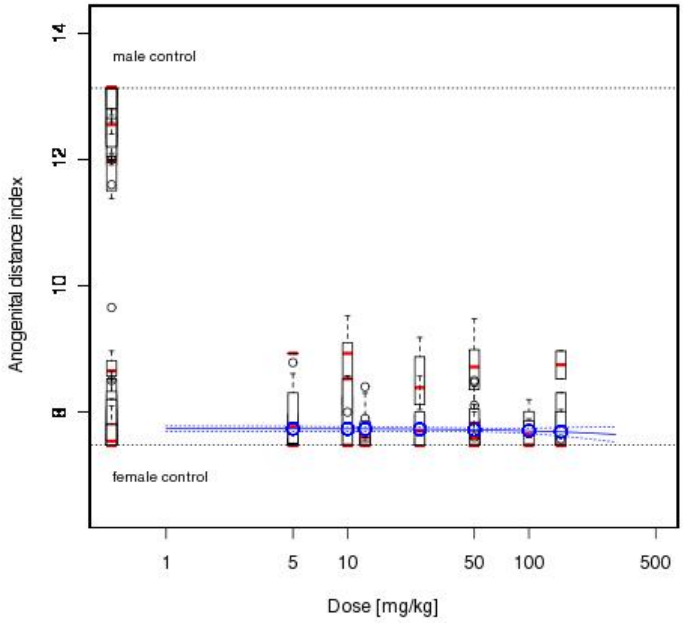
Mancozeb



Prochloraz



Procymidone



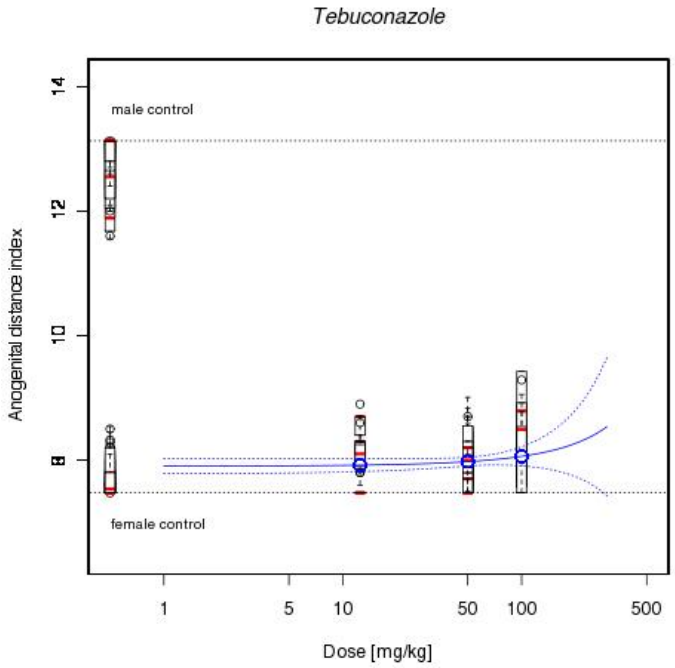


Figure 4.10: GEE fits of female AGDI for epoxiconazole, mancozeb, prochloraz, procymidone and tebuconazole.

The results in table 4.10 and figure 4.11 show that both independent actions and dose-additivity predictions highly underestimates the effect of the pesticide mixture. So, although no statistical effect is seen for epiconazole, mancozeb, procymidone and tebuconazole individually, they do seem to contribute to the effect in the mixture groups.

Table 4.10: Predictions of mixture response doses and their 95% confidence intervals (CI).

Relative AGDI	10%	25%	50%	75%	90%
AGDI (rounded)	8.04	8.89	10.30	11.72	12.56
Mean dose (observed)	38.0	104.0	170.0	236.0	302.0
95% CI (observed)	[34.0-42.0]	[92.0-124.0]	[147.0-202.5]	[199.0-275.0]	[245.0-346.5]
Mean dose (additivity)	224.0	720.6	1217	1714	2210
95% CI (additivity)	[217.8-230.3]	[709.6-731.6]	[1200-1234]	[1691-1737]	[2180-2239]
Mean dose (independent)	209.5	694.5	1228	1750	2255
95% CI (independent)	[202.9-216.1]	[687.9-701.1]	[1211-1245]	[1726-1773]	[2226-2285]

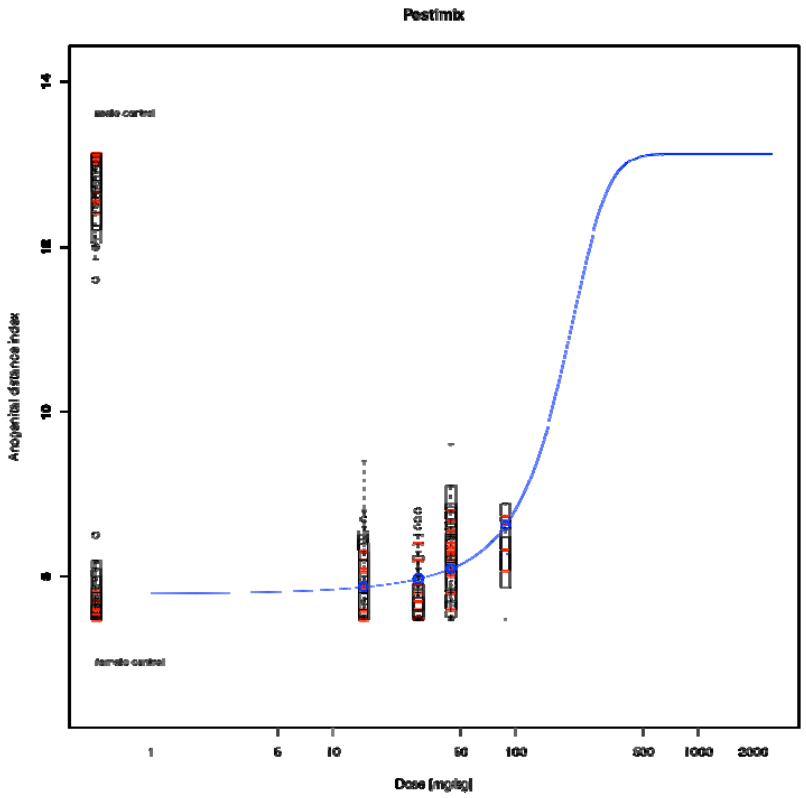


Figure 4.11A: GEE fit of procymidone, +/- 2 standard errors are illustrated with dashed lines (in blue). The observations are shown as box plots of each litter with red marks at the litter means.

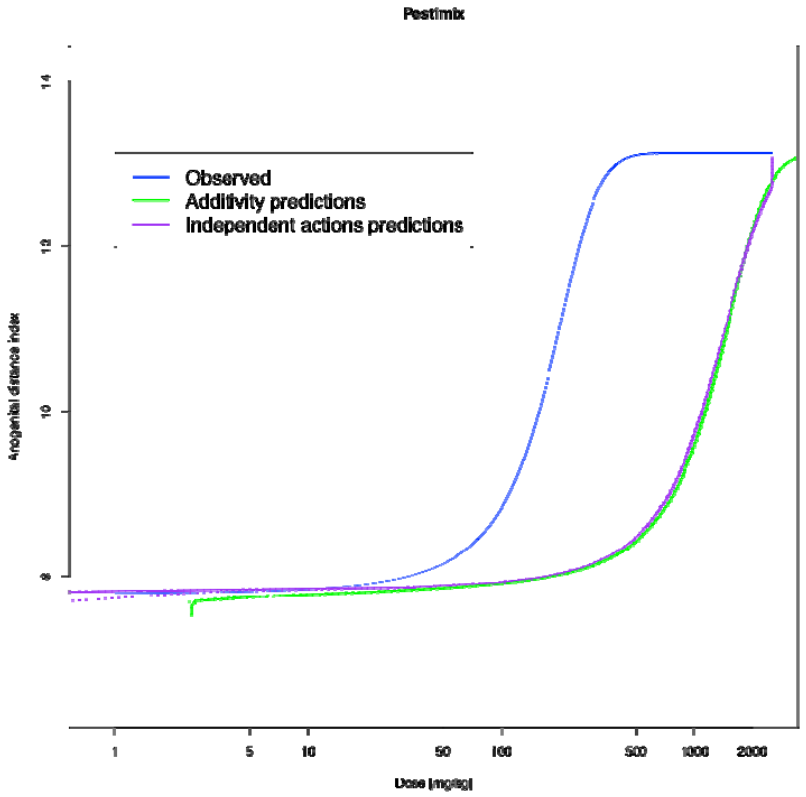


Figure 4.11B: The observed and predicted does effects of GL. The dose-additivity predictions of the effect doses are in green, the independent action predictions are in purple, and the observed curve in blue. In dotted lines +/-2 standard errors are illustrated.

In table 4.11, the effect doses for prochloraz are shown.

Table 4.11: Effect doses of prochloraz. Mean doses when using Monte Carlo sampling.

Relative NR	10%	25%	50%	75%	90%
Prochloraz	44.81	144.1	243.5	342.8	441.9

Using winsorization, the estimated effect doses were slightly higher for the low effect doses and slightly lower for the high effect doses (data not shown). However, the overall picture was the same.

4.4 Summary of main findings

For nipple retention in male offspring, the prediction of the mixture effect based on dose-additivity was in agreement with the observed effects at low doses, as there were no statistically significant differences between the predicted and the observed effect doses. However, dose-additivity underestimated the effects for the high doses of the mixture. The independent actions predictions were seen to even stronger underestimate the effects for high doses, and overestimated the effect for the low doses. A similar picture was seen for the endpoint gestation length, where the dose-addition prediction at low doses gave a good prediction of the observed effects. Both dose-additivity and independent action underestimated the mixture effects at high doses. However, the underestimation was generally smaller for the dose-additivity prediction than the independent action prediction.

Both independent action and dose-additivity predictions highly underestimated the effect of the pesticide mixture on female AGDI. So, although no statistical effect is seen for epiconazole, mancozeb, procymidone and tebuconazole individually, they do seem to contribute to the effect in the mixture. Based on the findings in the experimental study as well as our extensive historical control data showing no effect on female AGDI for procymidone and mancozeb, it is considered most likely that only epoxiconazole and tebuconazole has contributed to the effect in the mixture groups.

5 Chemical analysis of pesticides in rat blood

5.1 Introduction

In the studies described in chapter 3 the dams were exposed to procymidone, tebuconazole, epoxiconazole, mancozeb or prochloraz or a mixture of the five pesticides.

Since the pups were exposed indirectly via placenta and milk, it was of interest to know what the levels of exposure were to the pups. In order to investigate the internal doses of these compounds, both in dams and after birth in the pups, blood was drawn from the animals when they were sacrificed and chemical analysis was performed. Since it was shown that mancozeb did not affect the most relevant endocrine disrupting effects of the pesticide mixture (i.e. nipple retention and gestation length) and since mancozeb analysis would require a method which would be non-compliable with the determination of the other fungicides, the serum levels of mancozeb were not determined.

In the literature it is primarily methods for the quantitative determination of organochlorine pesticides which are available. To our knowledge there is no literature on quantitative analysis of procymidone, epoxiconazole, tebuconazole or prochloraz in serum, plasma or blood available in the literature.

The aim of the present study was to determine the levels of the four pesticides in serum from rat dams and their pups after exposure to either a mixture of all four or one of the four pesticides. The effect of different doses on the serum levels was also studied. It is possible that some compounds are absorbed more efficiently and transferred to the foetus and excreted via the breast milk more efficiently than others. The exposure to several compounds might also results in higher serum levels of some or all of the pesticides, if the excretion and/or metabolism of these are dependent on the same mechanisms or pathway and these therefore are overloaded.

To meet these aims a GC-MS/MS (gas chromatography–mass spectrometry) method for the quantification of procymidone, epoxiconazole, tebuconazole and prochloraz in serum from rat dams and pups was developed and validated. Only small amounts of blood can be drawn from rat pups, and blood samples were also needed for others purposes in the study. It was therefore a requirement that the method should only require small amounts of serum (~0.2 ml). As a ubiquitous requirement the method should also be simple, cost effective and sensitive.

5.2 Methods

5.2.1 Blood samples

All of the blood samples were taken 1-5 hours after the morning dosing of the maternal animals. The dams were exposed to a mixture of the fungicides procymidone, epoxiconazole, tebubonazole and prochloraz at different levels. Mancozeb was also in the mixture, but was not measured.

In the range finding study in pregnant animals, the low dose dams received a mixture of 12.5, 3.75, 12.5, and 8.75 mg/kg bw/day, of the four chemicals respectively. The high dose dams received a mixture of 25, 7.50, 25 and 17.5 mg/kg bw/day, respectively. The dams and the pups were sacrificed on day 13 after birth and blood samples were taken for chemical analysis. The number of samples used for pesticide analysis included: 2 from dams and 17 from pups representing 4 litters at the low mixture dose and 3 from dams and 8 from pups representing 4 litters at the high mixture dose.

In the extended mixture study, some of the pups from each litter were sacrificed on PD 16 and blood samples were taken. The samples were pooled within litters. The samples from the groups exposed to two doses of each of the pesticides were used and there were 2 to 7 samples per group. The samples from the mixture groups were not available for pesticide analysis as they were used for analysis of hormones. Samples were not taken from dams as they had to be kept until weaning of their offspring on PD 22.

5.2.2 Chemicals

Standards of the four pesticides were purchased from Dr. Ehrensdorfer (Augsburg, Germany) and were of ≥96% purity. Acetonitrile (HPLC grade) were purchased from Rathburn (Walkerburn, Scotland). Magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) was purchased from J.T. Baker (Bie & Bertsen, Herlev, Danmark).

Stock solutions of pesticide of 1 mg/ml were prepared in toluene and kept at -18°C. Standard mixtures were prepared by volumetrical dilution of the stock solutions with acetonitrile. Matrix matched calibration standards in the concentration range 0.0002 to 0.087 µg/ml were prepared by diluting the standard mixtures with acetonitrile to concentrations equal to the double of the intended concentrations and further diluting them 1:1 with cucumber matrix. The cucumber matrix was obtained by dispersive solid phase extraction of a homogenised organic cucumber sample (10 g) with acetonitrile (10 ml) and 4.0 g $MgSO_4$, 1 g NaCl, 1 g Na_3Citrate dehydrate and 0.5 g Na_2HCitrat sesquihydrate followed by clean up with PSA (25 mg PSA and 150 mg $MgSO_4$ per ml extract)(Comite Europeen de Normalisations, 2008**).**

5.2.3 Extraction procedure

0.20 g of serum was weighed into an Eppendorf tube. QC samples were spiked with 25 µl of an appropriate standard solution to obtain spiking levels of 0.002, 0.007, 0.022 and 0.218 µg/g . After addition of 1.00 ml of acetonitrile the tubes were briefly shaken before 0.1 g NaCl and 0.2 g $MgSO_4$ were added. Then the samples were extracted by shaking the tubes vigorously

by hand for 1 min. The extracts were centrifuged (Eppendorf miniSpin plus) for 5 min. at 9660 g.

Aliquots of the supernatants were transferred to auto sampler vials and added cucumber matrix (1:1) and mixed thoroughly. The sample extracts are mixed with cucumber matrix because we have experienced that the recoveries are improved considerately. Cucumber matrix is a matrix well suited for analyte protection and does not introduce significant problems with interfering matrix. The matrix components can interact with the analyte or active sites in the inlet or GC system and hereby protect the analyte from degradation and/or binding to the active sites and hereby increase the instrumental response and the overall recovery. Both calibration standards and sample extracts were therefore mixed with cucumber matrix, to obtain the same matrix effect. The extracts were analysed by GC-MS/MS.

5.2.4 Analysis

The GC-MS/MS analysis of rat serum and cucumber were performed on a Quattro Micro Tandem GC-MS/MS (Water, USA). The system consisted of a PAL-GC Autosampler, an Agilent GC 6890N and a Quattro Micro Tandem mass spectrometer. The GC was equipped with a Gerstel PTV injector for large volume injection. The injector was programmed from initially 30°C for 0.8 min to 290°C at a rate of 480°C per min and held for 2 min before further incensement to 310°C at a rate of 720°C per min. Purge time was 3.05 min and aliquot of 5 µl extract was injected. The oven programmed from initially 60°C for 1 minute increasing to 180°C at a rate of 30°C per min and then further increased to 260°C at a rate of 4°C per min. This temperature was maintained for 20 min. Chromatography was performed on a RESTEK, Rxi®-5ms, 30 m., 0.25 mmID, 0.25 µm df. The mass spectrometer was operated in the electronic ionization mode (EI, 70 eV). The temperature of the transfer line and ion source was set at 250°C and 180°C, respectively. Multiple reaction monitoring (MRM) was used to perform mass spectrometric quantification of the pesticides. The monitored MRM transitions, retention times and scan times are listed in table 5.1.

Quantification is based on calibration curves of eight matrix matched standard solutions, covering the relevant concentration range.

Table 5.1. Analyte retention time, scan time, and quantifier and qualifier MRM transitions.

Pesticide	Retention time (min)	MRM transitions, m/z (collision energy, eV)	
		Quantification	Identification
Procymidone	15.61	283 > 96 (5)	283 > 254 (10)
Tebuconazole	19.97	250 > 125 (15)	125 > 89 (10)
Epoxiconazole	20.57	192 > 138 (10)	206 > 165 (5)
Prochloraz	25.08	180 > 138 (10)	310 > 268 (5)

5.2.5 Validation

The analytical method was validated to demonstrate the specificity, precision, accuracy and lower limit of determination of measurements. Specificity was established by the lack of interference peaks at the retention time for the four relevant pesticides.

Linearity was tested at eight levels of concentrations covering a range of 0.0002–0.087 µg/mL. The calibration curves were established by plotting the peak area of the four pesticides at the eight different calibration levels. The regression parameters of slope, intercept and correlation coefficient were calculated by linear least square regression (1/x2 weighting).

The recovery or accuracy of the method was determined recovery test samples (n=2) in double replicates at 0.0022, 0.0072, 0.022 and 0.218 µg/ml of each of the four pesticides in cucumber and rat serum. Chromatograms for samples spiked with 0.007 µg /ml for procymidone, tebuconazole and epoxiconazole, and 0.022 µg/ml for prochloraz are shown in figure 5.1. Since the availability of rat serum was limited the first four series of were performed on spiked cucumber matrix, and then the performance was confirmed by analysing one series spiked rat serum.

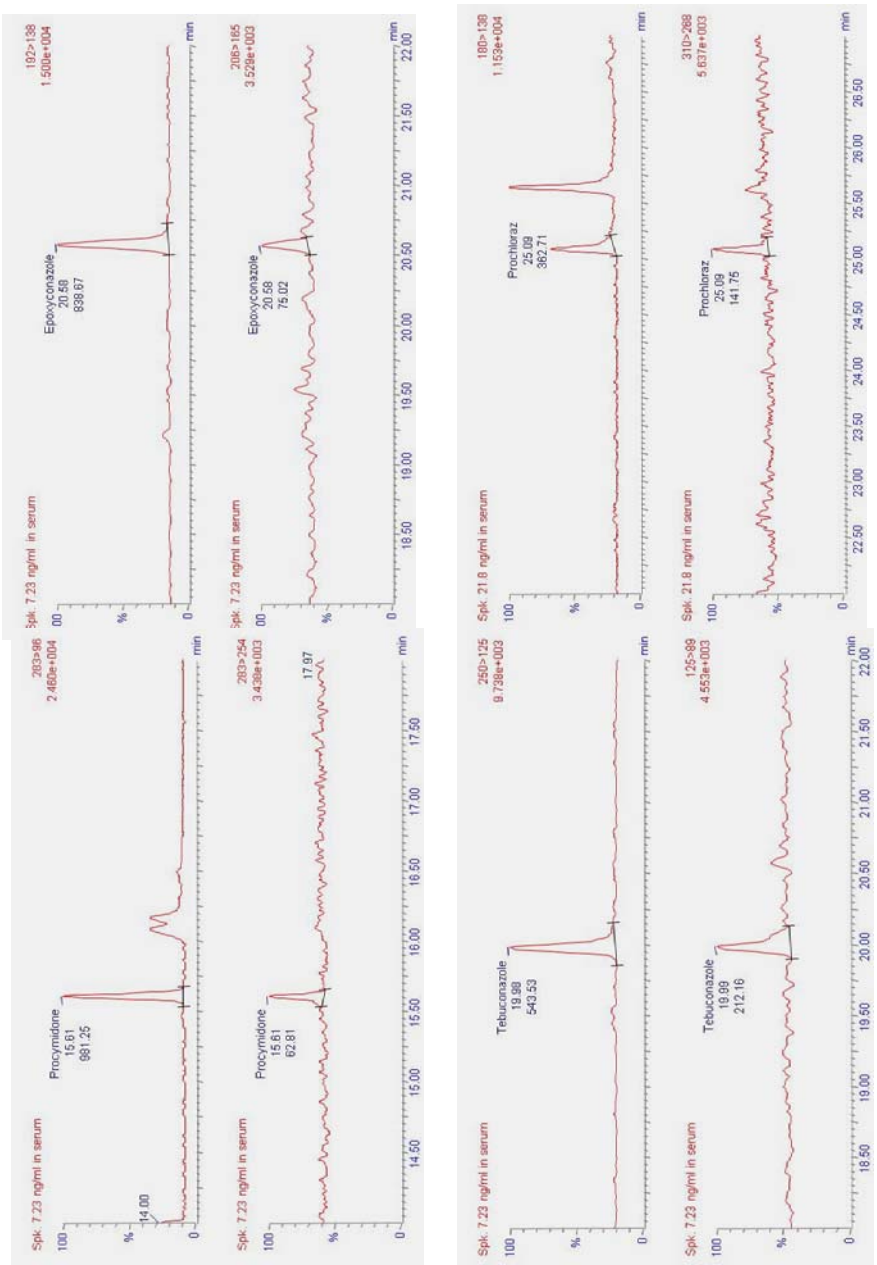


Figure 5.1: Chromatograms of QC serum samples spiked with 0.007 µg/ml for procymidone, epoxiconazole and tebuconazole and 0.022 µg/ml for prochloraz (matrix matched with cucumber extract 1:1 prior to analysis).

Mean recovery, relative repeatability (RSDr) and relative internal reproducibility (RSDR) and limit of determination (LOD) were calculated for each of the four compounds. RSDr and RSDR were calculated in accordance with ISO 5725-2 (1994) and the LODs were calculated as three times the standard deviation. The results are presented in table 5.2, however results not meeting the acceptance criteria are not presented.
The validation was found acceptable if the following criteria were meet: 1) the mean accuracy at each spiking level was in the range 70-110%; 2) the calculated LOD was close to the lowest spiking level, and 3) the relative repeatability and internal reproducibility were less than 20 for the highest spiking levels and 22% for the three lower spike levels. Accepting a relative repeatability and reproducibility of ≤20% is in accordance with Horwitz (1982). However, the acceptable relative standard deviations proposed by Horwitz are concentration dependent and the Horwitz equation therefore gives unacceptably high values at analyte concentrations below 100 µg/kg. The acceptance criteria of ≤22% for the three lower spiking levels in the present work are in accordance with the recommendation by Thompson (2000).

The described method was found valid for the quantification of procymidone, epoxiconazole, tebuconazole and prochloraz in rat serum. LOD for procymidone, epoxiconazole, tebuconazole and prochloraz were 0.0006 µg/g, 0.002 µg/g, 0.002 µg/g and 0.011 µg/g, respectively. A recovery of 122% for procymidone, at the lowest spiking level, was found acceptable because the relative standard deviation and internal reproducibility were low.

Table 5.2. Results of the method validation; spike levels, mean recoveries, relative repeatability (RSDr,%), relative reproducibility (RSDR, %) and limit of determination (LOD, µg/g) for procymidone, epoxiconazole, tebuconazole and prochloraz.

Spike level, µg/g	0.0022	0.0072	0.022	0.22
Procymidone				
Recovery, %	122	100	96	97
RSD_r, %	6	6	5	4
RSD_R, %	8	8	7	8
LOD, µg/g	0.0006			
Epoxiconazole				
Recovery, %		100	92	94
RSD_r, %		9	6	6
RSD_R, %		9	9	8
LOD, µg/g		0.002		
Tebuconazole				
Recovery, %		97	100	96
RSD_r, %		13	10	4,4
RSD_R, %		11	12	8
LOD, µg/g		0.002		
Prochloraz				
Recovery, %			100	111
RSD_r, %			14	9
RSD_R, %			17	14
LOD, µg/g			0.011	

5.3 Results

5.3.1 Range finding study 2 (mixture exposure)

The results obtained by the analysis of the serum samples from the range finding study are presented in (table 5.3). Both the high and low mixture dose resulted in quantifiable levels of all four fungicides in the serum of dams. The serum levels of procymidone where highest (medians 1.88 and 4.81 µg/g serum for low and high dose, respectively). The serum levels of epoxiconazole and tebuconazole where lower (medians of 0.677 to 2.47 µg/g serum), about half of the procymidone levels, but similar to each other. The levels of prochloraz, in the serum from the dams, were 40 to 60 times lower than the procymidone levels.

Procymidone were also detectable in all serum samples from the pups. The levels of epoxiconazole were above LOD in all pup serum samples from the high dose group and most samples from the low dose group (10 of 17 samples). The levels of tebucoanzole were only quantifiable in a few samples, i.e. 2 out of 8 samples and 3 of 17 samples from the low and high dose group, respectively. As the dose of epoxiconazol was only around 30% of the tebuconazol dose the results indicate that a larger fraction of the administrated dose of epoxiconazole, than of tebuconazole, is transferred un-metabolised from the dams to the pups. Prochloraz was below LOD in all pup serum samples. Thus, the serum levels of procymidone were significantly higher than the levels of the three other fungicides in both dams and pups.

In table 5.5 (row 1 to 4) are some factors presented, which describes the relations between the levels found in serum from the animals from the high dose groups and the low dose groups in the range finding study. From these factors it can be seen that the levels of procymidone in serum from pups and dams increased three-fold by a two-fold increase in the dose (table 5.5, rows 1 and 2). The median serum levels of procymidone, found in the dams, were about 9 to 10 times higher than in the pups of the corresponding groups (table 5.5, rows 3 and 4).

By doubling the mixture dose the serum levels of epoxiconazole in dams and pups where increased by a factor of 4 and 2, respectively. The serum levels for rat dams were 100 to 190 times higher than in the pups following exposure to the low dose and the high dose, respectively. Doubling the mixture dose resulted in four times higher serum levels of tebuconazole for the dams but in no clear increase in the serum level for the pups. The median serum level for tebuconazole in dams were 240 to 1200 times higher than in the pups, the highest values found for the high dose group. Only a few results for tebuconazole were above, but still close to the LOD, whereby the factors calculated are associated with some uncertainty. However, even if all positive results, i.e. also those <LOD (8 and 6 results for pups in the low and high dose group, respectively) are included in the calculation, the factors for tebuconazole in row 3 and 4 in table 5.5, is found to be 570 and 2400. So even with the two later values being higher than those presented in the table, these still indicate that the serum levels of tebuconazole is much lower in serum from the pups than in serum from the dams.

All pup serum levels of prochloraz were below LOD and factors are therefore not presented for this compound. From the factor two stated in row 1 in table

5.5 for prochloraz it is indicated that the serum levels for the dams are doublet by doubling the mixture dose.

Table 5.3. Range-finding study 2 in pregnant animals. Serum levels of procymidone, epoxiconazole, tebuconazole and prochloraz, (µg/g) in rat dams and pups (mean values) exposed indirectly via the dams, which are exposed to a mixture of all four compounds during both pregnancy and lactation period. n.a.: not applicable.

	Serum levels (µg/g serum)			
Pup/dam (litter no., pup no.)	Procymidone	Epoxiconazole	Tebuconazole	Prochloraz
	Low mixture dose (mg/kg bw/day)			
	12.5	3.75	12.5	8.75
Dam (9)	1.80	0.755	0.531	0.032
Dam (10)	1.96	0.659	0.822	0.062
Median (Dams)	1.88	0.707	0.677	0.047
Female (9, 3)	0.111	0.009	0.003	<LOD
Female (9, 4)	0.363	0.015	0.003	<LOD
Female (10, 4)	0.153	<LOD	<LOD	<LOD
Female (10, 6)	0.224	0.002	<LOD	<LOD
Female (14,3)	0.149	0.003	<LOD	<LOD
Female (16,3)	0.356	0.007	<LOD	<LOD
Male (9, 7)	0.209	0.007	<LOD	<LOD
Male (9, 8)	0.209	0.007	<LOD	<LOD
Male (9, 9)	0.226	<LOD	<LOD	<LOD
Male (9, 10)	0.358	0.007	<LOD	<LOD
Male (9, 11)	0.136	<LOD	<LOD	<LOD
Male (10, 3)	0.138	<LOD	<LOD	<LOD
Male (10, 4)	0.216	<LOD	<LOD	<LOD
Male (10, 6)	0.239	<LOD	<LOD	<LOD
Male (14, 4)	0.209	<LOD	<LOD	<LOD
Male (14, 5)	0.244	0.003	<LOD	<LOD
Male (16, 3)	0.888	0.018	0.003	<LOD
Median (Pups)	0.216	0.007	0.003	n.a.
	High mixture dose (mg/kg bw/day)			
	25	7.50	25	17.5
Dam (17)	12.7	5.90	7.69	0.279
Dam (23)	5.68	2.90	2.93	0.102
Dam (24)	3.93	2.04	1.14	0.051
Median (Dams)	4.81	2.47	2.04	0.077
Female (23, 3)	0.481	0.010	<LOD	<LOD
Female (24, 3)	0.600	0.016	<LOD	<LOD
Male (17, 4)	1.180	0.040	0.003	<LOD
Male (17, 5)	0.897	0.003	<LOD	<LOD
Male (22, 4)	0.414	0.015	<LOD	<LOD
Male (22, 5)	0.742	0.017	<LOD	<LOD
Male (23, 3)	0.541	0.003	<LOD	<LOD
Male (24, 2)	0.407	0.017	0.002	<LOD
Median (Pups)	0.570	0.016	0.002	n.a.

The analysis of serum samples from the range finding study therefore indicate that; 1) procymidone results in the highest internal dose for rat dams; 2) the internal dose of epoxiconazole and tebuconazole were comparable for dams though the exposure to epoxiconazole was lower; 3) the internal dose of tebuconazole in pups where lower than for epoxiconazole; 4) by doubling the dose the internal dose of the dams are triplet for procymidone and quardruplet for epoxiconazole and tebuconazole but only doublet for prochloraz; 5) doubling the dose also resulted in a tripling of the internal dose

of procymidone in pups, whereas the internal dose of epoxiconazole in pups where doublet and for tebuconazole the internal dose for the pups seemed unaffected by the increased dose; 6) the degree to which the compounds where transferred to the pups via dams milk seems to be descending in the following order procymidone > epoxiconazole > tebuconazole.

5.3.2 Extended mixture study (single compound exposure)

Dams in the extended mixture study were exposed to one of the four fungicides at two different doses, the high dose being four times higher than the low dose (table 5.4) throughout pregnancy and during the subsequent lactation period. The results presented in table 5.4 are obtained by the analysis of pooled serum samples, for which serum from both male and female pups within the same litter have been pooled. Thus, each result represents an average serum concentration for two to six pups.

The levels of the four different fungicides in the pup serum varied considerably. The highest levels were, also in this study, found for procymidone. The median level of procymidone in the high dose and low dose group was 0.217 µg/g serum and 0.098 µg/g serum, respectively.

The levels of epoxiconazole and tebuconazole in serum from the high dose group were similar. The medians were for both compounds about 0.004 µg/g. In serum from the low dose group however the contents of tebuconazole was <LOD (0.002 µg/g), whereas contents of epoxiconazole above LOD were found in 3 of 6 litters exposed to expoiconazole with a median (of results above LOD) of 0.003 µg/g. Even though the administrated dose of epoxiconazole is three times less than tebuconazole, the serum levels of the two are similar. Based on the results of the low dose groups, a three times lower dose of epoxiconazole may even result in higher serum levels than tebuconazole.

None of the serum samples from litters exposed to prochloraz contained detectable levels. However, since the LOD for prochloraz (0,011µg/g), obtained by the method used in the present study, is relatively high, residues of prochloraz may occur in the serum. In fact small peaks were observed in some samples but the contents were too low to quantify.

If comparing the serum levels found in serum from pups in the low dose group with the same from the high dose group it is indicated that by quadrupling the doses the serum levels of the pups are increased by a factor of two for procymidone and epoxiconazole. The serum levels of tebuconazole on the other hand seems in this single chemical exposure study to be more dose dependent, since a factor of four is indicated. This factor is however uncertain, because is it found by comparing a median serum level of the high dose group (0.004 µg/g) with the median of the low dose group (0.001 µg/g), which is obtain from values all below LOD (table 5.5, row 5).

5.3.3 Possible mixture effect on internal doses?

In order to conclude on whether or not the internal doses of the individual compounds are altered when exposed to a mixture than when administrated one compound at a time, a comparison of the results from the range finding study with the results from the extended mixture study was done. Since dam serum was not available for the extended mixture study, comparisons could only be made for pups.

From table 5.3 and table 5.4 it can be seen, that the doses of the pesticides in the low dose groups were the same in the two studies, whereas the highest doses of the single pesticides in the extended mixture study were twice as high as the doses in the high dose mixture group in the range-finding study. By comparing the medians yielded from the two sets of data, it is indicated that the exposure of the dams to all four fungicides results in higher serum levels of procymidone and epoxiconazole in the pups, than when dams are exposed to one compound at a time. For the low dose groups the median serum levels of procymidone and epoxiconazole for the pups are twice as high when administrated as a mixture, compared to when administrated as single compounds. For tebuconazole, a possible effect of co-administration is not as apparent. The same serum level (0.001 µg/g serum) is found if all values, including those <LOD, are included in the calculation of the median serum levels. All values of prochloraz are below LOD in all pup-serum samples in both studies.

Table 5.4. Extended mixture study. Average serum levels (µg/g) for rat pups within each litter and median serum level for the whole group exposed indirectly via the dams during pregnancy and lactation period with one of the four fungicides at either high level (15-50 mg/kg bw/day) or low level (3.75-12.5 mg/kg bw/day). *: result is the average of double determinations.

Dosis respons study	Doses (mg/kg bw/day)	Litter	Serum conc. (µg/g serum)	Median of positives
Procymidone	50	49*	0.252	
		98	0.217	
		100	0.192	0.217
	12.5	45	0.041	
		95	0.165	
		96*	0.096	
		146	0.099	
		193*	0.114	
		195	0.061	0.098
Epoxiconazole	15	28	0.004	
		77	0.003	
		175*	0.009	0.004
	3.75	23	<LOD	
		124	0.003	
		171*	0.007	
		172	<LOD	
		173	0.002	
		174	<LOD	0.003
Tebuconazole	50	141	<LOD	
		144	0.004	
		191	0.003	
		192	0.012	0.004
	12.5	40	<LOD	
		91	<LOD	
		139	<LOD	
		187	<LOD	
		188	<LOD	
		189	<LOD	
		190	<LOD	n.a.
Prochloraz	35	88	<LOD	
		137	<LOD	
	8.75	86	<LOD	
		134	<LOD	
		136	<LOD	
		181	<LOD	
		184*	<LOD	n.a.

Table 5.5. Factors calculated by division of different median serum level values to aid the interpretation of the results obtained from analysis of serum samples from the range-finding study (mixed exposure) and the extended mixture study (single chemical exposure)

			Procy-midone	Epoxi-conazole	Tebu-conazole	Prochlo-raz
Mixed exposure	1	Dams high/ Dams low	3	4	4	2
	2	Pups high/ Pups low	3	2	1	n.a.
	3	Dams low/ Pups low	9	100	240[a]	n.a.
	4	Dams high/ Pups high	10	190	1200[b]	n.a.
Single chemical exposure	5	Pups high/ Pups low	2	2	(4)[c]	n.a.

[a]: Based on only three results for pups, other 14 results were <LOD.
[b]: Based on only two results for pups, other 6 results were <LOD.
[c]: A median value of 0.001 µg/g serum for tebuconazole in pups from the low dose group has been used based on six results <LOD for the calculation of the factor.

The factors presented in table 5.5, describes by which factor the serum levels are increased by increasing the exposure levels. By doubling the mixture dose in the range-finding study, the serum levels in pups were increased by a factor of three for procymidone, a factor of two for epoxiconazole, and the levels of tebuconazole seemed unchanged. The latter factor for tebuconazole is only based on a few results above the LOD, however even if all positive results though <LOD were included in the calculation a factor of 1 was obtained.

Thus, these results indicate a mixture effect for procymidone and epoxiconazole, since higher serum levels of these two compounds are found if they are administrated as part of a mixture, than when administrated as a single compound.
This effect has not been indicated for tebuconazole. For tebuconazole the median serum levels for pups in the two low dose groups in the two studies are <LOD and close to LOD (0.003 µg/g serum), thus no clear mixture effect was seen.

It is not possible to evaluate whether or not there is a mixture effect associated with exposure to prochloraz. Further studies needs to be performed with a more sensitive method.

5.4 Summary of main findings

Procymidone, epoxiconazole, tebuconazole and prochloraz could be detected in all serum samples from dams and as expected the serum levels in the dams were generally higher than those in the pups, exposed indirectly via maternal milk. Procymidone occurred at highest levels. In dams, epoxiconazole and tebuconazole resulted in similar levels when administrated as part of a mixture, but the transfer of epoxiconazole, to the pups, was indicated to be more efficient than for tebuconazole. Also by single compound exposure the serum levels of epoxiconazole were found to be higher than for tebuconazole, even though the dose of epoxiconazole were lower than for tebuconazole. Prochloraz was detected in some pup serum samples but all levels were below LOD.

By comparing the results obtained from the analysis of the pup serum samples from the two studies mixture effects are indicated for procymidone and epoxiconazole. The same dose resulted in higher internal dose in the pups of the two compounds when administrated as part of a mixture than when administrated alone.

The GC-MS/MS method for the determination of procymidone, epoxiconazole, tebuconazole and prochloraz in serum was developed and validated. The method is quick and easy to perform. The accuracy and precision obtained was acceptable.

The sensitivity was found acceptable for procymidone, tebuconazole and epoxiconazole. The sensitivity for prochloraz was relatively low, resulting in a LOD of 0.01 µg/g, and quantification of the low levels found in the pup serum samples was not possible.

6 *In vitro* studies

6.1 Introduction

The objective of performing the ***in vitro*** studies was to evaluate the usability of these methods for predicting the ***in vivo*** effects of the pesticides as well as the mixture. The outcome of such a retrospective comparison would be of value for future evaluations of the predictability of ***in vitro*** data. Overall, application of predictable ***in vitro*** assays may lead to faster and cheaper data generation for evaluation of chemicals and a reduction of the number of experimental animals that have to be used for risk assessment of chemicals.

The H295R cell line has been shown to express all the key enzymes for steroidogenesis as well as having the ability to produce the steroid hormones found in the foetal and adult adrenal cortex (Gazdar et al., 1990). The National Food Institute, DTU, has participated in the validation of this ***in vitro*** assay guided by OECD Validation and Management Group for Non-Animal Testing (VMG NA) (Hecker et al., 2007). Changes in the production of steroid hormones will be compared to the effects found in rodent studies.

The androgen reporter (AR) gene assay is used for the detection of chemicals' ability to interact with the AR as either agonists or antagonists. The AR reporter gene assay utilizes the fact that the AR is a transcription factor that induces transcription of target genes after binding to an androgen response element (ARE) in their promoter. In this assay Chinese Hamster Ovary (CHO) cells are co-transfected with the human androgen receptor expression vector. The ARE is linked to the gene of the easily measurable protein, the firefly luciferase, making the promoter active and luciferase expression AR regulated. In general, reporter gene assays provide a quick and accurate tool to determine and quantify hormone-induced transcriptional activity (Vinggaard et al., 1999, Andersen et al., 2002).

The T-Screen assay is an ***in vitro*** assay, which can be used for the detection of agonistic and antagonistic properties of compounds at the level of the thyroid receptor (TR). The assay is a proliferation assay based on the growth of the rat pituitary tumor cell line GH3 and growth is dependent on 3,3',5-triiodo-thyronine (T3). The cells express high numbers of TRs. The growth stimulatory effect of T3 is mediated by these specific, high-affinity TRs that upon binding of THs bind to TREs in the cell nucleus ultimately leading to gene expression (Hinkle et. al 1986). The T-screen assay can be used for ***in vitro*** detection of agonistic and antagonistic properties of compounds at the level of the TR, but it will not catch effects on TH levels resulting e.g. impairment in TH synthesis pathways (Hohenwarter et al., 1996).

6.2 Materials and Methods

6.2.1 Mixture for *in vitro* assays

The so-called 'PestiMix' for ***in vitro*** studies was designed based on the mixture applied for the ***in vivo*** studies. The ratio of the 5 pesticides was:

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

6.2.2 Androgen receptor reporter gene assay

The ability of the pesticides to activate the AR and to inhibit androgen-induced activation of the AR was tested in the AR reporter gene assay as described by Vinggaard et al., 1999, with modifications. Briefly, CHO cells (7000 cells per well) were transfected for 5 h with a total of 75 ng cDNA/well consisting of the expression vector pSVAR0 and the MMTV-LUC reporter plasmid (both kindly provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam) in a ratio of 1:100 using 0.3 µl of the transfection reagent FuGene (Roche Diagnostics, Mannheim, Germany). Cells were washed and test compounds were added with or without 0.1 nM of the AR agonist R1881 and were tested in eleven concentrations within the range of 0.03-30 µM in triplicates. OHF was included in every experiment as a positive control. Luciferase activity was measured directly using a Lumistar Galaxy luminometer by automatically injection of 40 µl substrate containing 1 mM luciferin (BD Sciences Denmark, Brondby, Denmark) and 1 mM ATP (Roche Diagnostics) in lysis buffer and the chemiluminescence generated from each well was measured. The observations were normalized to correct for interassay variation.

Cytotoxicity experiments were performed as described above, except that the cells were transfected with the constitutively active androgen receptor 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.

6.2.3 Mixture model predictions

The observed ***in vitro*** AR antagonistic effect of the mixture was compared with the dose/concentration addition (DA) and independent action (IA) model predictions.
Experimental data from independent experiments were pooled by normalizing values in each separate experiment to control (vehicle-control). Experimental data for single chemicals were then plotted into an X-Y graph in Graph pad prism, with Log10 of chemical concentration on the X-axis and effect in percent of control on the Y-axis. A four parameter logistic non-linear regression curve fit was then applied to data from each chemical. The following formula describes this curve fit:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Maximum, minimum, Hill slope and EC50 values for each chemical were obtained. And transferred to a spread sheet (Excel 2010).

DA was then calculated using the following formula:
$EC\boldsymbol{x}_{mix} = (\sum p_i / EC\boldsymbol{x}_i)^{-1}$

$EC\boldsymbol{x}_{mix}$ is the effect concentration of the mixture provoking x% effect
P_i is the constant proportion of the total concentration $\boldsymbol{c}_{mix}$ at which chemical I is present in the mixture. $EC\boldsymbol{x}_i$: is the equivalent effect concentrations i.e. those concentrations/doses of chemicals that alone would cause the same quantitative effect x as the mixture. The individual effect concentration EC***xi*** was derived from the four parameter logistic curve fit of the experimental data of each chemical, using information on the Maximum, minimum, Hill slope and EC50 values obtained in GraphPad Prism.

IA was calculated using the following formula

$E(c_{mix}) = 1- \Pi (1 - (E(C_i))$

$E(C_i)$ was in parallell to EC***xi*** for DA, calculated by use of Maximum, minimum, Hill slope and EC50 values obtained in GraphPad Prism.

Experimental data for the mixture was then plotted in GraphPad Prism with a concomitant 95% confidence limit band. Predictions for DA and IA obtained in Excel were then plotted on the same graph, and visually compared to the 95% confidence band of the experimental data.

6.2.4 T- screen for thyroid receptor activity

The rat pituitary cell line GH3 obtained from American Type Culture Collection (ATCC) were cultured in a humid atmosphere at 37°C and 95% air/5% CO_2 in phenol-red free DMEM/F12 (Gibco-Invitrogen, Paisley, UK) supplemented with 1% Antibiotic/Antimycotic (PSF) and 10% (vol/vol) fetal calf serum (FCS) (Gibco). The cells were grown in medium containing 10% (vol/vol) triiodothyronine (T_3)-depleted dextran-charcoal treated FCS (DC-FCS). Removal of thyroid hormone from DC-FCS was performed by treatment with Bio-Rad AG-1X8 resin (Copenhagen, Denmark) as described in Samuels et al. (1979) modified by changing the resin three times after 6, 18, and 6 h of centrifugation at 37°C by centrifugation and adding fresh resin. Finally the last resin was removed by centrifugation at 27,000 g for 10 min and the serum was sterilized by filtration through a 0.2 µlm polyethersulfone (PES) filter. Forty-eight hours prior to plating the cells into 96-well microplates (Costar, Fisher Scientific Biotech Line) standard culture media was changed to test media containing 10% (vol/vol) T_3- and thyroxine (T_4)-depleted DC-FCS. After 24 h medium was changed and after 48 hr GH3 cells were harvested and seeded in 96-well black, clear bottom microplates, 50 µl of cell suspension at a density of 2500 cells per well and 50 µl of test compound per well.

All compounds were tested in triplicate (0, 0.01, 0.375, 1, 3, 10, and 30µM) and were tested both in the absence or presence of 0.22nM T3 (T3-EC50) to test for agonistic and antagonistic potency. Control wells contained cells and test medium with the same amount of DMSO [0.1%] as the exposed cells. After incubation for 96 h, cell growth was measured using the dye resazurine (O'Brien et al., 2000). 100 µl, of a 0.005 mg/ml resazurine solution in PBS, was added to each well, and plates were incubated 3 h at 37°C, protected from light. Fluorescence was measured (excitation wavelength 560

nm/emission. 590 nm) on a Wallac Victor 1420 multilabel counter (PerkinElmer Life Sciences). Generally, testing included 6 samples, i.e. the mixture and the 5 individual pesticides, at several dose levels and triplicate assessment of the endpoints in the assays.

6.2.5 Steroid synthesis in the H295R cell line

The pesticides were investigated for effects on the production of estradiol, progesterone, and testosterone in the human adrenocortical carcinoma cell line H295R (ATCC, CRL-2128) as described previously (Hecker et al., 2010). In brief, cells were seeded at a density of 3×10^5 cells/well in 24-well culture plates (Costar 3524, Corning, NY, USA) with DMEM/F12 medium (Gibco, Paisley, UK) supplemented with 2.0% Nu-serum (BD Sciences Denmark) along with 1% ITS + premix (containing 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml BSA and 5.35 µg/ml linoic acid; BDSciences Denmark) and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2/air.

The pesticides were added (1ml) to the cells in triplicates at six concentrations. Control wells contained the same amount of DMSO (0.1%) as exposed cells. After 48 h incubation the medium was removed and stored at −20 °C until analysis of hormones. The levels of estradiol, progesterone, and testosterone in the cell medium were measured using commercial hormone kits from WallacDelfia (PerkinElmerDenmark, Hvidovre, Denmark). Hormone levels were normalized to control levels.

Cytotoxicity was recognized by adding 1ml 0.05 mg/ml resazurin (Sigma–Aldrich) to the cells and after 2–4 h of incubation then measuring the fluorescence (excitation 560 nm, emission 590 nm) on a Wallac multilabel 1420 counter.

6.3 Results

6.3.1 Androgen receptor data

All pesticides except for mancozeb exhibited AR antagonism ***in vitro*** (Fig 6.1). The lowest observed effect levels (LOEC) and cytotoxic concentrations are shown in the table 6.1. The ranking of potencies for AR antagonism was: Mixture ≈ Procymidone > Prochloraz ≈ Epoxiconazole > Tebuconazole. As the content of procymidone in the mixture constitutes only approximately 1/3 of the total mixture dose, these results indicate combination effects on AR antagonism ***in vitro***.

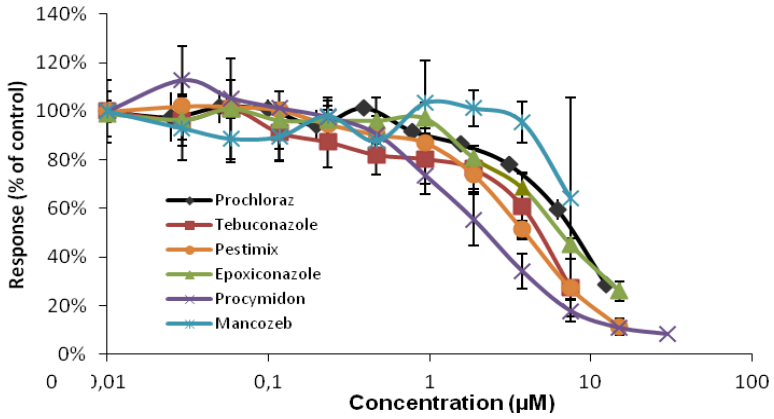


Figure 6.1. Androgen receptor antagonism of single pesticides and the mixture *in vitro.*

6.3.2 Mixture model predictions on AR antagonist mixture data

As an experiment we tried to model the AR antagonist data to evaluate if the effects of the mixture could be predicted by dose/concentration addition (DA) and/or independent action (IA). As can bee seen in figure 6.2 the ***in vitro*** AR antagonistic effect of the mixture could be equally predicted by the DA and the IA model as both were situated within the 95% confidence band of the experimental mixture data.

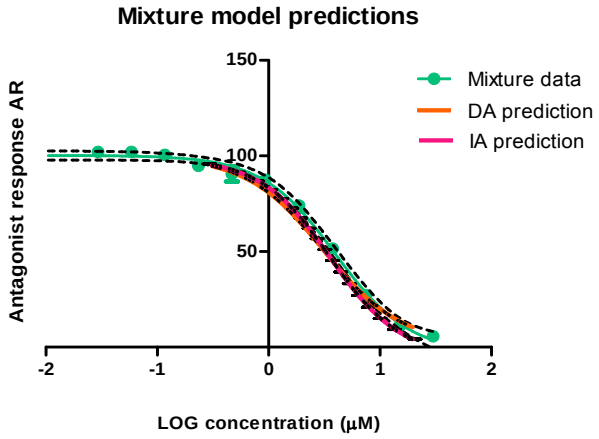


Figure 6.2. Mixture model predictions. The *in vitro* AR antagonistic effect of the mixture was compared with the dose/concentration addition (DA) and independent action (IA) model predictions.

6.3.3 H295R Steroid synthesis data

Prochloraz, tebuconazole, epoxiconazole and the mixture was shown to reduce the testosterone level in the cells, whereas procymidone and mancozeb had no effect (fig. 6.3). Prochloraz, epoxiconazole, tebuconazole and the mixture also reduced estradiol levels, whereas procymidone and mancozeb at higher concentrations above 10 µM caused increased estradiol levels (fig. 6.3). Generally the pesticides and the mixture caused an increase in progesterone levels, although for the mixture and for procymidone the effect was only seen at the highest concentrations tested (fig. 6.3).

In general the effects of prochloraz was most pronounced on all three hormones.

6.3.4T-Screen data

As can be seen in figure 6.4, triiodothyronine (T_3) induced dose-dependent proliferation of the GH3 cells as expected.

No agonistic effect was observed for the pestimix (fig. 6.5.a). A weak antagonistic effect of the pestimix was observed at concentrations at and above 3.13µM (fig 6.5.b). However at concentrations above 25µM cytotoxic effects were reported.

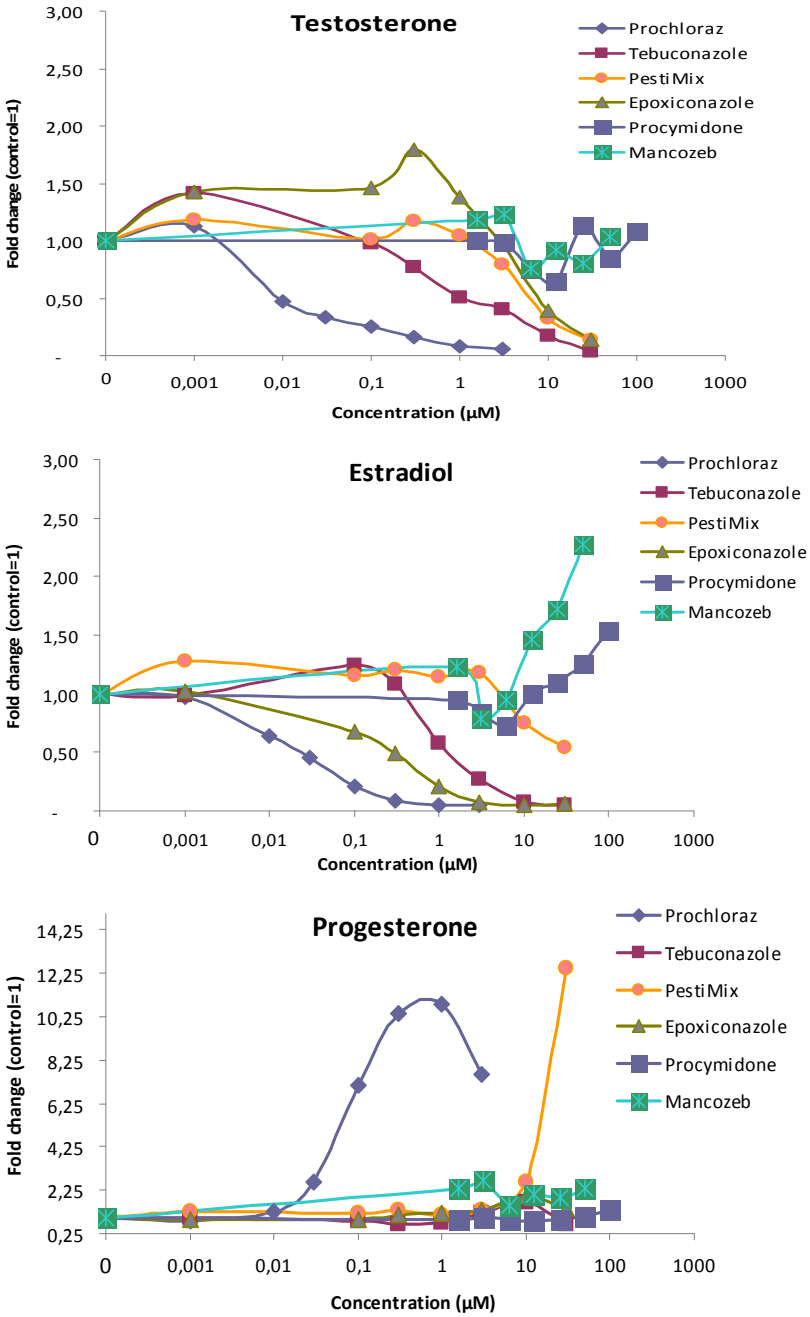


Figure 6.3 Changes in levels of testosterone (top), estradiol (midle) and progesterone (bottom) in the H295 steroid synthesis assay, after exposure to increasing concentrations of the 5 pesticides singly or in combination.

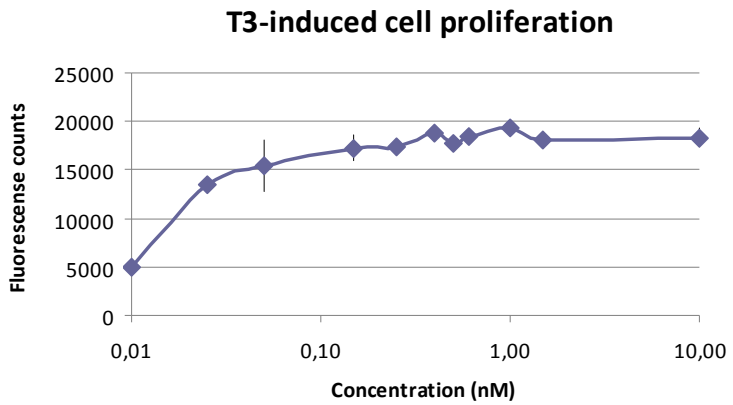


Figure 6.4. T_3-induced cell proliferation.

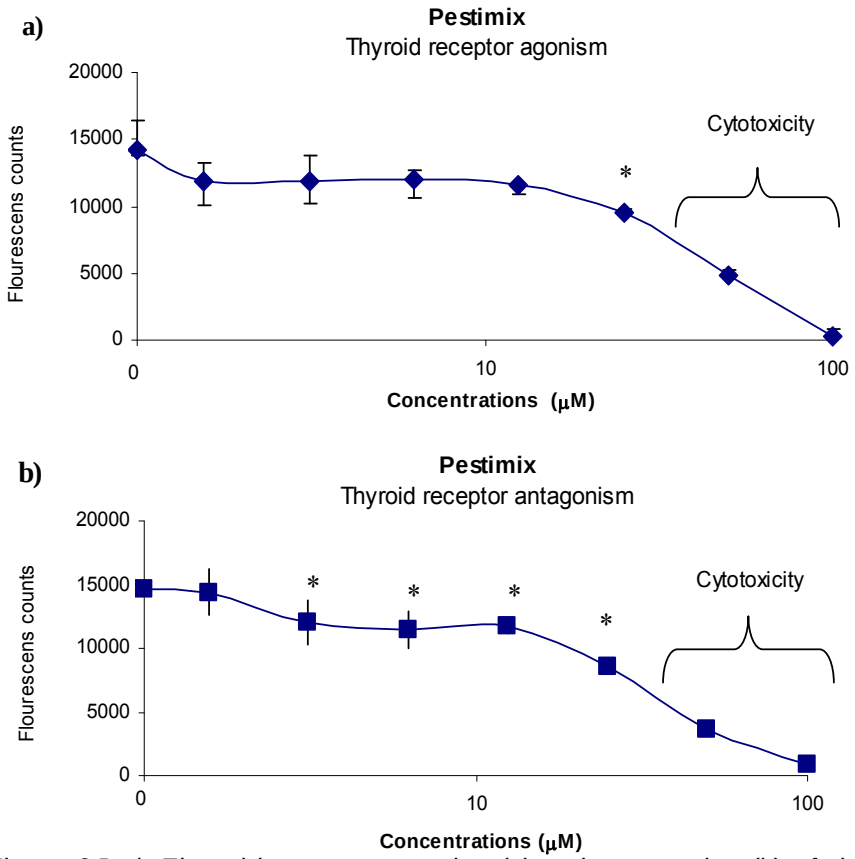


Figure 6.5 a,b. Thyroid receptor agonism (a) and antagonism (b) of the pesticide mixture in the T-Screen assay. The data represent mean±SD from one representative experiment out of 3 independent experiments.
* Statistically significantly different ($p \leq 0.05$) from the respective controls

6.4 Summary of main findings

A summary is shown in table 6.1. All pesticides except for mancozeb exhibited AR antagonism ***in vitro***. The ranking of potencies for AR antagonism was: Mixture ≈ Procymidone > Prochloraz ≈ Epoxiconazole > Tebuconazole. As the content of procymidone in the mixture constituted only approximately 1/3 of the total mixture dose, these results indicated combination effects on AR antagonism ***in vitro***.

In the H295 steroid synthesis assay prochloraz, tebuconazole, epoxiconazole and the mixture was shown to reduce the testosterone level in the cells, whereas procymidone and mancozeb had no effect. Prochloraz, epoxiconazole, tebuconazole and the mixture also reduced estradiol levels, whereas procymidone and mancozeb at higher concentrations caused increased estradiol levels. In general the effects of prochloraz was most pronounced on all the hormones.

In the T-Screen no agonistic effect was observed for the pestimix. A weak antagonistic effect of the pestimix was observed at concentrations at and above 3.13µM. However at concentrations above 25µM cytotoxic effects were reported

Table 6.1 Summary of the *in vitro* effects

	AR assay		Steroid hormone production (H295R cell assay)			T-Screen assay (cell proliferation)	
	Anta-gonistic effect LOEC[a]	Ago-nistic effect	Estradiol	Testosterone	Progesterone	Anta-gonistic effect	Ago-nistic effect
Procymidone	0.9 µM	-	↑#	-	↑*	-	-
Prochloraz	1.9 µM	-	↓	↓	↑	-	-
Epoxiconazole	1.9 µM	-	↓	↓	↑	-	-
Tebuconazole	3.8 µM	-	↓	↓	↑	↓	-
Mancozeb	-	-	↑#	-	-	↓	↓
Pestimix	0.5 µM	-	↓	↓	↑	↓¤	-

a) LOEC: Lowest Observed Effect Concentration
- : no effect.
: at concentrations ≥12.5 µM
* : at concentrations ≥ 50 µM
¤ : at ≤ 25 µM, at higher concentrations the mix was cytotoxic

7 Survey of pesticide intake

7.1 Introduction

Risk assessment of pesticides is currently based on the no observed adverse effect levels (NOAELs) for effects of single compounds. However, humans can be exposed to various chemicals at the same time that contribute to a cumulative effect in the same individual. The Regulation EC No. 396/2005 from European Parliament and the Council Exposure require that cumulative and synergistic effects of pesticides are considered. With the aim to address this issue EFSA has submitted a ´Opinion on the Risk Assessment for a Selected Group of Pesticides from the Triazole Group to Test Possible Methodologies to Assess Cumulative Effects from Exposure through Food from these pesticides on human Health (EFSA 2009a). In this opinion the probabilistic approach together with the Relative Potency Factor approach (RPF) as described by Wilkinson et al. (2000) was used to estimate the cumulative exposure to the group of triazoles which have a common mode of action. As also described in the ´Opinion of the Scientific Panel on Plant and their residues on a request from EFSA to evaluate the suitability of exciting methodologies´ (EFSA 2008) and Boon et al. (2004) the probabilistic approach is needed when addressing the exposure from different pesticides contained in several crops simultaneously. RPFs, which express the relative toxic potency of each compounds compared to an index compound (IC) are used to normalize the residue levels for all compounds in the group.

Cumulative dietaty exposure calculations using the probabilistic approach together with the RPF approach have also been performed on other pesticides, which have a common mode of action, e.g. the organophosporus and carbamate pesticides which both are acetyl cholin esterase inhibiting compounds (Jensen et al., 2009, Boon et al., 2003, Caldas et al., 2006). As part of this project the effects of cumulative exposure from the five endocrine disrupting pesticides prochloraz, procymidon, tebuconazole, epoxiconazole and mancozeb were investigated. Prochloraz, procymidon, tebuconazole and epoxiconazole all caused effect on nipple retention in male offspring based on a group mean offspring of 1 nipple, whereas prochloraz, tebuconazole and epoxiconazole all increased the gestation length based on a group mean increase of 0.2 days (Jacobsen et al., 2010). For the effect on nipple retention, RPFs for procymidone, tebuconazole and epoxiconazole were derived from these effect studies using prochloraz as index compound whereas RPFs for tebuconazole and epoxiconazole were derived for the effect on gestation length using prochloraz as index compound. Mancozeb did not affect nipple retention or gestation length so the intake calculations did not include this substance.

The aim was to estimate the cumulative dietary exposure to prochloraz, procymidone, epoxiconazole and tebuconazole which all affect nipple retention and the cumulative dietary exposure to prochloraz, epoxiconazole and tebuconazole which all affect gestation length. To do this we used the probabilistic approach together with the RPF approach. Since the two common endocrine disrupting effects on nipple retention and gestation length are of interest for women in the childbearing age the intake calculations have

been performed for women in the age of 15-50 years old. For comparison the intake calculations have also been performed for children and the general population.

7.2 Materials and methods

Intake estimates were based on consumption data obtained from the Danish National Dietary Survey 2000-2002 conducted by the Department of Nutrition, Technical University of Denmark (Andersen et. al. 2005). The survey included 4068 participants aged 4-75 years old. The participants could be characterized as close to representative for the Danish population. Children aged 4-6 years accounted for 231 participants and women in the age 15-50 years accounted for 1176 participants. Dietary intake was recorded for 7 consecutive days. A pre-coded food diary that included answering categories for the most commonly eaten foods and dishes in the Danish diet was used. The questionnaire was organised according to a typical Danish meal pattern (breakfast, lunch, dinner and snacks). Portion sizes were given in predefined household measures or estimated from photographs. Data was collected over the whole year to take into account seasonal variation in dietary habits. Self-reported body weight was obtained from a personal face-to-face interview and used in the calculations.

Residue data for the three pesticides prochloraz, procymidone and tebuconazole were obtained from the Danish pesticide monitoring programme during the period 2006-2009 while residue data for epoxiconazole were obtained from the Swedish pesticide monitoring programme during the period of 2007-2009. Altogether the residue data included 3203 samples of fruits and vegetables. The sampling plan is set up to ensure compliance with the maximum residue limits set in the legislation and with regard to which commodities that contributes most to the intake. Only commodities with at least one detectable residue above the limit of quantification (LOR) were included in the calculations. The selected pesticides were found in 21 commodities.

As described in the opinion from EFSA "Opinion on the Risk Assessment for a Selected Group of Pesticides from the Triazole Group to Test Possible Methodologies to Assess Cumulative Effects from Exposure through Food from these pesticides on human Health" (EFSA 2009a) relative potency factors (RPF) are derived for the common effects for the pesticides.

In this study two common effects were seen in the rat studies. One effect was increased gestation length based on a group mean increase of 0.2 days and the other effect was nipple retention in male offspring based on a group mean offspring of 1 nipple. The RPF approach was used for assessing the cumulative exposure. The concentrations of the four pesticides were expressed in equivalents of one index compound, and then summed. The relative potency factors were developed based on the low end of the dose response curves in the rat studies, as humans are expected to be exposed to low doses. Prochloraz was selected as index compound for the exposure calculation because this compound affects both gestation length and nipple retention in male offspring. The residue concentration for a given pesticide is multiplied by the RPF value for this substance to give an equivalent content of the index compound. The cumulative exposure of the four pesticides adjusted to the index compound prochloraz can then be summed and compared to the Derived No Effect Level (DNEL) for the index compound, which were

obtained from the ***in vivo*** rat studies. Relative potency factors for the two effects are shown in tables 7.1 and 7.2.

To get the most realistic picture of the intake of pesticides, processing factors are an important issue to address. The pesticides included in the intake calculations were found among other commodities in mandarins, orange, grape fruits, lemon, water melon for which processing factors as peel/pulp distribution are normally applied in a refined exposure calculation. In this study a processing factor of 0.1 was applied as provided from the report of the Danish monitoring programme (Poulsen et al., 2004).

Table 7.1. Derived No Effect Level and RPFs for increased gestation length based on a group mean increase of 0.2 days.

	Common effect			
Compound	ED_GL0,2 BMD[1] (mg/kg bw)	Uncertainty factor (UF)[2]	DNEL mg/kg bw	RPF
Prochloraz	25	(3 x 100 x 3,3)[3] = 1000	0.025	1
Tebuconazol	47.1	1000	0.0471	0.5
Epoxiconazol	5.1	1000	0.0051	4.9

[1)] Bench Mark Dose
(UF)[2] Uncertainty factor
[3]Default uncertainty factor on 100 for NOAEL, 3 for effect only close to NOAEL and 3.3 for taking into consideration intake of other endocrine disrupting substances.

Table 7.2. Derived No Effect Level and RPFs for increased nipple retention in male offspring based on a group mean increase of 1 nipple.

	Common effect			
Compound	ED_NR BMD[1] (mg/kg bw)	Uncertainty factor (UF)[2]	DNEL mg/kg bw	RPF
Prochloraz	25	(3 x 100 x 3,3)[3] = 1000	0.025	1
Tebuconazol	42.9	1000	0.0429	0.6
Epoxiconazol	36.9	1000	0.0369	0.7
Procymidone	7.14	1000	0.00714	3.5

[1)] Bench Mark Dose
(UF)[2] Uncertainty factor
[3] Default uncertainty factor on 100 for NOAEL, 3 for effect only close to NOAEL and 3.3 for taken into consideration intake of other endocrine disrupting substances.

Levels below the limit of reporting (LOR) can be handled in different ways. In theory, samples with levels below the LOR could contain residues below the LOR but higher than zero. The question is therefore which value is the best to use in the calculation, e.g. LOR, ½ LOR or zero. For comparison both ½ LOR and zero was used in the cumulative exposure calculations for pestimix causing nipple retention.

The cumulative acute exposure was calculated using the Monte Carlo Risk Assessment program (MCRA 6.2), an internet-based program developed by Biometris and RIVM – National Institute for Public Health and the Environment (RIVM), Bilthoven, UR, the Netherlands (deBoer et al., 2007). The calculations were performed as follows. A person was randomly selected out of the consumption database. The consumption of each commodity that person has eaten in one day was multiplied with randomly selected normalised residue concentrations in the residue database. The intake was summed over foods giving an empirical estimate of the acute cumulative intake distribution. This was performed 100.000 times for both the children, women in the child bearing age and the general population giving a probability distribution for the pesticide intake. No variability factors were used in the calculation. All

estimates of possible intakes were adjusted for the individual body weights for each consumer. The exposures were specified at percentiles P50, P90, P95, P99, P99.9 and P99.99 and compared to the DNEL for prochloraz. The percentile 99.9 was used as reference point. The uncertainties in the exposure calculations at each percentile expressed as a 95 % confidence interval were estimated using the Bootstrap technique (Manly 1998, Vose 2000, Caldas et al., 2006). The uncertainty at the different percentiles estimated by this technique is due to sampling uncertainty of the original data set (Manly 1998, Boon et al., 2004).

7.3 Results

7.3.1 Food consumption and residue data

Table 7.3 shows a summary of the food consumption data from the nationwide survey. Carrot followed by cucumber, tomato, oranges and sweet pepper had the highest mean consumption levels for both populations, all consumers and the women (total population, both consumers and non-consumers) of the foods that contained at least one positive level of the pesticides addressed. Melon followed by table grapes/peaches and tomato had the highest mean consumption on consumption days only (included only days with consumption) for both women in the child bearing age and the general population. The highest percentage of consumption days was seen for exotic fruit, cucumber, tomato and sweet pepper for both women in the child bearing age and the general population.

Table 7.3. Summary of food consumption data from Danish national dietary survey 2000-2002.

Crop	Mean consumption (g/day of total survey days)[a]		% consumption days[b]		Mean consumption (g/day, consumption days only)	
	Women 15-50 years[c]	General population[d]	Women 15-50 years	General population	Women 15-50 years	General population
Apricot	0.17	0.1	0.3	0.4	27.7	29
Bean with pod	0.12	0.1	5.1	4.8	2.3	2.5
Carrot	35.2	32	43	43	81.5	76
Cucumber	22.9	21.7	55.0	51.6	40.3	42.1
Dates, dried	0.15	0.13	0.3	0.3	50.7	46
Fruit exotic	0.42	0.41	83.3	81.7	0.5	0.5
Grapefruit	1.9	1.6	12.6	11	14.8	14
Leek	2.3	2.1	11.5	11	19.8	20
Lettuce	10.4	8.8	40.5	34	25.6	25.9
Lemon	1.3	1.0	5.9	4.3	21.9	21
Mandarin	6.6	5.7	12.6	11	52.4	51
Melon	8.6	7.2	4.0	3.6	215	213
Oranges, sweet	12.1	10	12.6	11	95.6	92
Peach, nectarines	5.0	3.5	4.9	3.8	101.7	95
Plum	1.3	1.4	2.1	2.3	56.1	65
Raspberries	0.14	0.17	1.3	1.6	11	10.7
Rhubarb	0.10	0.15	1.4	2.4	6.7	6.3
Strawberry	2.71	2.9	3.9	4.9	69.2	58.2
Table grapes	6.7	5.2	6.5	5.7	103	92.4
Sweet pepper	4.9	7.9	53.1	47	16.4	16

Tomato	22.7	20.9	64.8	59.8	35.1	35.0

[a] Included zero and non-zero consumption days.
[b] % of non-zero consumption days for consumers
[c] Women 15-50 years
[d] General population aged 4-74 years

Table 7.4. Summary of residue data of procymidone, prochloraz, tebuconazole analyzed in the Danish and monitoring programme during 2006-2009 and epoxiconazole analyzed in the Swedish monitoring programme during 2007-2009. Data are used in the exposure calculation to the effect nipple retention.

Commodity	Samples analyzed	Samples detected		Mean positive Concentration (mg/kg)
Apricot	1	1	(100%)	0.024
Bean with pod	97	8	(8.25)	0.216
Carrot	57	4	(7.0%)	0.023
Cucumber	81	5	(6.2%)	0.258
Dates, dried	1	1	(100%)	0.09
Fruit exotic	120	38	(31.6%)	0.411
Grapefruit	7	242	(2.9%)	0.127
Grape, table	21	454	(4.6%)	0.432
Leek	4	33	(12.1%)	0.022
Lemon	20	257	(7.8%)	0.028
Lettuce	6	68	(8.8%)	0.554
Mandarin	34	330	(10.3%)	0.043
Melon	2	108	(1.9%)	0.007
Oranges, sweet	10	277	(4.3%)	0.021
Peach, nectarines	49	256	(19.1%)	0.038
Plum	14	209	(6.7%)	0.163
Raspberry	3	18	(16.7%)	0.98
Rhubarb	1	1	(100%)	0.03
Strawberry	5	118	(4.2%)	0.048
Sweet pepper	12	220	(5.5%)	0.311
Tomato	8	135	(5.9%)	0.249

Table 7.5. Summary of residue data of prochloraz and tebuconazole analyzed in the Danish monitoring programme during 2006-2009 and epoxiconazole analyzed in the Swedish monitoring programme during 2007-2009. Data are used in the exposure calculation to the effect gestation length.

Commodity	Samples analyzed	Samples detected		Mean positive Concentration (mg/kg)
Apricot	1	1	(100%)	0.020
Bean with pod	7	56	(12.5 %)	0.023
Carrot	4	57	(7.0%)	0.019
Dates, dried	1	1	(100%)	0.075
Fruit exotic	37	102	(36.3%)	0.411
Grapefruit	7	242	(2.9%)	0.013
Grape, table	17	299	(5.7%)	0.047
Leek	4	33	(12.1%)	0.022
Lemon	20	257	(7.8%)	0.028
Mandarin	33	275	(12.0%)	0.045
Melon	1	56	(1.9%)	0.007
Oranges, sweet	10	277	(3.6%)	0.021
Peach, nectarines	48	223	(21.5%)	0.031
Plum	12	154	(7.8%)	0.027
Rhubarb	1	1	(100%)	0.03
Sweet pepper	1	53	(1.9%)	0.06
Tomato	3	74	(4.1%)	0.037

Table 7.4 shows the frequencies, mean concentration in samples with positive content found in the 21 different commodities of fruit and vegetables that contributed to the exposure to the effect of increased nipple retention.

Apricot, rhubarb and dates had the highest frequency (100%) due to residues in the only one sample taken. Hereafter, exotic fruits, including passion fruit, mango, guava, carambola, kaki, rambutan and kumquat had the highest detection frequency (31.6 %) followed by peach/nectarines (19.1 %), raspberry (16.7 %), leek (12.2 %) and mandarin (10.3%). Commodities with the highest mean concentration in samples with content higher than the level of reporting were exotic fruit, lettuce, grapes, cucumber, bean with pod and plums.

Table 7.5 shows the frequencies, mean concentration in samples with positive content found in the 17 different commodities of fruit and vegetables that contributed to the exposure to the effect on increased gestation length. Apricot, rhubarb and dates had the highest frequency (100%) due to residues in the only one sample taken. Hereafter exotic fruits, including passion fruit, mango, guava, carambola, kaki, rambutan and kumquat had the highest detection frequency (36.23%) followed by peach/nectarines (21.5 %), bean with pod (12.5%), leek (12.1 %) and mandarin (12.0 %). Commodities with the highest mean concentration in samples with content higher than the level of reporting were exotic fruit, date, table grape, mandarin, tomato and peach/ nectarin.

7.3.2 Cumulative exposure and risk assessment

Table 7.6 shows the cumulative exposure estimates for pestimix nipple retention and pestimix gestation length using prochloraz as index compound. Levels below the level of reporting were set to zero. The exposure calculated in µg/kg bw/day was compared to the derived no observed adverse effect level (DNEL) of 25 µg/kg bw/day for prochloraz. DNEL was the same for both effects. Exposure calculation is shown for children, women in the child bearing age and general population for both effects.

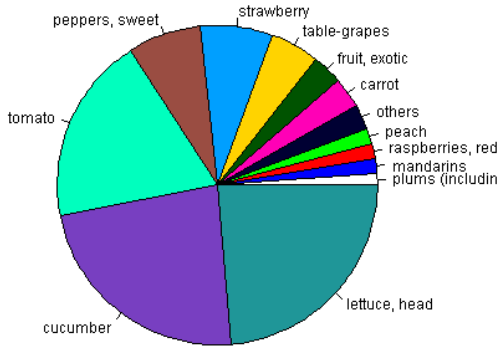
Table 7.6. Cumulative exposure calculations for two endocrine disrupting effects, i.e. nipple retention (NR) and gestation length (GL) using prochloraz as index compound

	Pestimix_NR		Pestimix_GL	
Percentile	% of DNEL	95 % confidence interval	% of DNEL	95 % confidence interval
Children 4-6 years				
95	0.9	(0.5-1.7)	0.2	(0.1-0.3)
99	5.3	(2.4-8.9)	0.7	(0.2-0.8)
99.9	19.9	(8.3-28)	1.9	(1.5-2.3)
99.99	60.1	(27-160)	5.1	(3.0-5.6)
Women 15-50				
95	0.5	(0.3-0.7)	0.1	(0.1-0.1)
99	2.2	(1.0-4.0)	0.3	(0.2-0.4)
99.9	8.9	(3.7-13.1)	0.9	(0.7-1.1)
99.99	34.8	(16-61)	2.2	(1.4-3.2)
General population 4-75 years				
95	0.4	(0.3-0.7)	0.1	(0.1-0.1)
99	2.3	(1.3-4.1)	0.3	(0.2-0.4)
99.9	10.2	(6.0-14.0)	0.9	(0.7-1.1)
99.99	35.0	(17-70)	2.4	(1.6-2.9)

Looking at nipple retention and using the percentile 99.9 as cut off percentile the exposure is estimated to be between 8.9 – 19.9 % of the DNEL of 25 µg/kg bw/day for the index compound prochloraz. For gestation length the exposure is between 0.9 – 1.9 % of the DNEL of 25 µg/kg bw/day for prochloraz.

7.3.3 Crops and pesticides that contributed most to the intake

Figure 7.1 shows which commodities that contributed most to the intake for both effects nipple retention and increased gestation length. The calculations are performed for the general population. As earlier mentioned women in the childbearing age is the group of interest in this study. As seen in table 7.3 and 7.6 the eating habit and exposure for women in the child bearing age and the general population was nearly the same. The general population includes 4 times more persons compared to the number of women in the childbearing age in the dietary survey. The calculations are therefore expected to be more robust using the general population. It is seen that the six commodities lettuce, cucumber, tomato, sweet pepper and strawberry contributes most to the intake when looking at the effect of increased nipple retention while the six commodities exotic fruit, carrot, tomato, mandarins, peach and table grapes contributed most to the cumulative intake, when looking at the effect on increased gestation length.



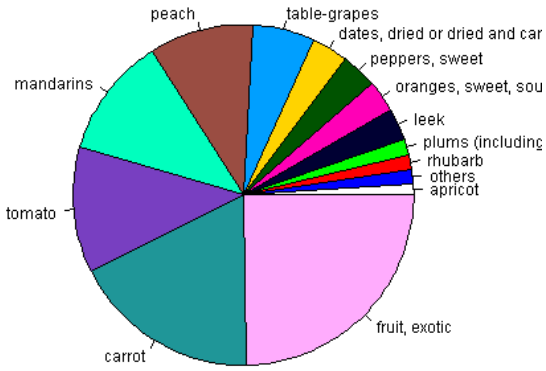


Figure 7.1. The commodities contribution to the exposure for the two effects nipple retention (upper figure) and gestation length (lowest figure). The figure are shown for the general population

Figure 7.2 shows which pesticides that contributed most to the exposure for the effect on nipple retention. The results are shown for the general population. Epoxiconazole was only found in one commodity (leek). Because of the very small contribution from this substance it is not shown in the figure. It is seen that procymidone contributes far most to the exposure. For gestation length tebuconazole and prochloraz contributed by the same quantity but as shown in table 7.5 the cumulative exposure was low.

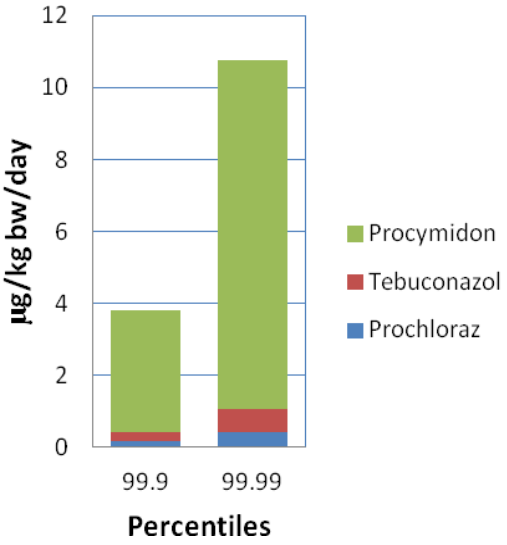


Figure 7.2. The pesticides that contributed most to the exposure for the effect on nipple retention shown at different percentiles.

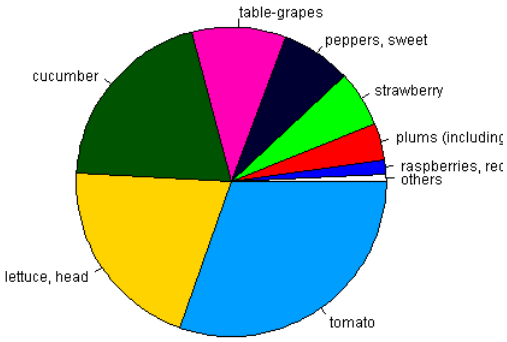
7.3.3.1 Nipple retention using RPF approach

Figure 7.3 shows the contribution to the intake calculations for procymidone that contributed most to the intake for the effect on nipple retention. Furthermore the uncertainties in the calculations are shown at different percentiles. The calculations have been performed for the general population.

7.3.4 Effect of using ½ level of reporting (LOR)

To study the impact of using ½ LOR such calculation was performed for the effect on nipple retention for the whole population. At the 99.9 percentile the cumulative intake was increased from 10.2 % to 10.7 % of the DNEL of 25 µg/kg bw/day for prochloraz.

Procymidone (RPF 3,5, DNEL 25 µg/kg bw/day)



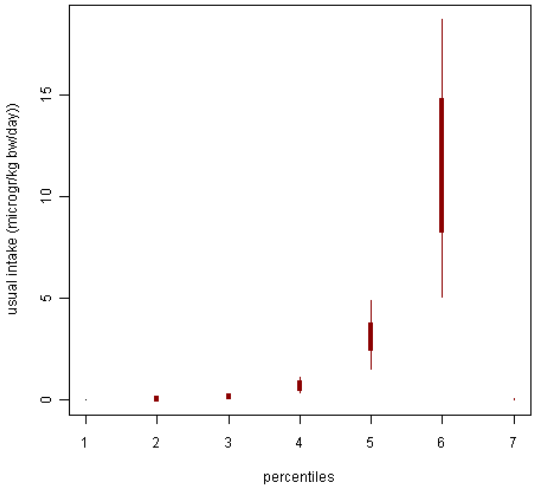


Figure 7.3. Commodities that contributes most to the intake and the exposure shown at different percentiles including uncertainty. Percentile points in graph correspond with 1: 50.00, 2: 90.00 3: 95.00, 4: 99.00, 5: 99.90, 6: 99.99.

7.3.5 Exposure of the Danish population to the pesticides

To get an overview of the exposure of the Danish population (4-74 years) to the endocrine disrupting pesticides, a recalculation from the results on exposure with regards to nipple retention was performed by dividing these data with their RFPs. Intake data for the mean and the 50th to the 99.99th percentiles are shown in table 7.7 in µg/kg bw/day for prochloraz, procymidone and tebuconazole. Mancozeb exposure was not calculated as this pesticide did not contribure to nipple retention or gestation length in the present study, while intake of epoxiconazol was not calculated as this pesticide was only found in one sample (one leek out of 14 tested leeks, and not in any

other tested fruits or vegetables) and therefore the exposure levels in the Danish population are probably almost non-existing.

Table 7.5. Exposure to prochloraz, procymidone and tebuconazole in the general Danish population, recalculated from exposure data used for nipple retention and subsequent division with the RPF

Percentil	Prochloraz µg/kg bw/day	Procymidone µg/kg bw/day	Tebuconazol µg/kg bw/day
50	0	0	0
90	0.0048	0.01075	0.00383
95	0.01101	0.04054	0.0209
99	0.04522	0.21341	0.12353
99.9	0.15857	0.97675	0.41347
99.99	0.40877	2.76001	1.075
Mean	0.00248	0.0103	0.00525

7.4 Summary of main findings

The cumulative exposure of the endocrine disrupting pesticides was assessed for women in childbearing age of 15-50 years in Denmark using the probabilistic approach together with the RPF approach. The pesticides did not have the same mode of action but had two common effects. Four of the pesticides had effects on nipple retention and three caused increased gestation length. Relative potency factors for the two common effects were derived from the extended mixture study in rats.

Despite the uncertainties in the calculations of the dietary cumulative intake of the endocrine disrupting pesticides included in this project, the results indicated that the cumulative intake of the pesticides is below 100 % of the DNEL of 25 µg/kg bw/day for prochloraz for both effects at the percentile 99.9 for women in the childbearing age between 15-50 years.

8 Overall discussion and conclusions

8.1 Mixture effects below NOAELs

This project generally aimed at exploring the hypothesis that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides could lead to adverse developmental toxicity effects.

The first of the objectives was therefore to investigate whether a mixture of environmentally relevant endocrine disrupting pesticides with dissimilar modes of action, caused adverse developmental toxicity effects, including long-term delayed effects, at dose levels below NOAELs for the individual pesticides. For this purpose, range-finding studies and a comprehensive extended mixture developmental toxicity study in rats were performed.

As can be seen in table 8.1 several of the investigated endocrine and reproductive endpoints were affected by a combination of the 5 pesticides at dose levels where the individual pesticides caused no or smaller effects. These endpoints included gestation length, nipple retention, weight of male reproductive organs (prostate, seminal vesicles, epididymis and LABC) and genital malformations.

In the extended mixture study, gestation length was significantly increased in the two highest mixture groups and in the group exposed to the highest dose of epoxiconazole, but not in any other groups. The mixture doses consisted of each of the pesticides at 17% or 25% of their NOAEL for effects on gestation length based on our previous studies. As the highest dose of epoxiconazole given alone was 4 times higher that the dose included in the highest mixture group, these results show combination effect of the pesticides at dose levels where the individual pesticides caused no effects. This combination effect was actually observed already in the first range-finding study in pregnant animals, where the findings clearly showed that the combined exposure induced severe effects manifested as dystochia (impaired parturition) and high perinatal pup mortality. In the rangefinding study, the mixture doses consisted of each of the pesticides at their NOAEL or 75% of their NOAEL for effects on gestation lengths based on our previous studies.

Mixture effects on gestation length have to our knowledge not been studied by others meaning that this is a new finding. The mixture effect is probably due to the presence of the three azole fungicides in the mixture, prochloraz, epoxiconazole and tebuconazole, which have previously been shown to elicit such effects on gestation length (Noriega et al., 2005; Vinggaard et al., 2006; Taxvig et al., 2007). Prolongation of the gestation period may possibly be due to an increase in progesterone in the dams as suggested for prochloraz, epoxiconazole and tebuconazole (Vinggaard et al., 2005a; Taxvig et al., 2007). The dystochia and pup mortality seen in the range finding studies have previously been observed as common effects of several of the azole fungicides (Wolf et al., 1999, Moser et al., 2001, Noriega et al., 2005, Taxvig et al.,

2007). The mode of action for these effects may be similarly mediated via effects on progesterone and if that is the case these azole fungicides should be considered as similarly acting. However, further data are needed to make firm conclusions. Neither mancozeb nor procymidone have previously been shown to affect pregnancy length or perinatal survival at the doses studied (Metzdorff et al., 2007; Axelstad et al., 2011).

The number of nipples (aerolas) in male offspring in the extended mixture study was significantly higher than in controls in all three mixture groups, at the highest dose of mancozeb, prochloraz and tebuconazole and both doses of procymidone. However, the effect of the high dose of mancozeb (25mg/kg) is numerically quite small. As our earlier study of mancozeb including larger groups and higher dose levels (up to 100 mg/kg) has shown no effect on nipple retention (table 2.4), we find that the significant effect in this study is likely to be a random finding (Axelstad et al., 2011). For the other pesticides, similar effects have to some extent been seen for the individual pesticides in our earlier studies and by others (Ostby et al., 1999; Wolf et al., 1999; Noriega et al., 2005; Vinggaard et al., 2005a; Laier et al., 2006; Taxvig et al., 2007; Hass et al., 2007; Christiansen et al., 2008, Christiansen et al., 2009). No statistically significant effects of mancozeb, prochloraz and tebuconazole were found at the low dose level similar to the dose included in the highest mixture group, whereas procymidone caused a significant effect (table 8.1). Since the magnitude of the effect in the highest mixture group was significantly higher that the effect induced by procymidone alone at the dose level included in the highest mixture, the results indicate mixture effects for this endpoint. Mixture effects on this endpoint have been shown earlier after exposure to other mixtures of anti-androgens in several rat studies by us and others (Hass et al., 2007; Rider et al., 2008, Christiansen et al., 2009).

In the female offspring, significantly longer anogenital distance index (AGDI) was seen in the two highest mixture groups. A similar effect was seen in female offspring given the dose levels of epoxiconazole, prochloraz and tebuconazole included in the highest mixture group. No effect was seen in female offspring dosed with a four times higher dose of epoxiconazole and consequently the finding at the low dose of epoxiconazole may be a random finding or the lack of effect at the high dose may be due to the limited group size. However, the high doses of both prochloraz and tebuconazole did cause increased female AGDI. The effect observed in the mixture groups is evaluated most likely to be caused by the combined exposure to the three azole fungicides as earlier studies in our laboratory have also documented similar effects of prochloraz, epoxiconazole and tebuconazole (Laier et al., 2006; Taxvig et al., 2007). The increased AGD observed in the female offspring may be caused by increased progesterone levels in the dams (Willingham et al., 2006). The progesterone levels were however not measured in the present study in dams during gestation or around the time of delivery and we are therefore not able to evaluate if progesterone levels were correlated with the AGD effect in our study.

As can be seen in table 8.1, statistically significant effects on male reproductive organ weight and genital malformations were seen in the highest mixture group, whereas no significant effects were seen for the single compounds. On PD 16, reduced weights of epididymis, prostate and seminal vesicle were seen in the highest mixture dose group. Epididymis weight was lower than controls in all three mixture groups although this was only statistically significant in the low and high mixture groups. None of the

pesticides caused statistically significant effects on these endpoints when given alone at a dose similar to those included in the highest mixture group or a 4 times higher dose. Therefore, these results show mixture effect at dose levels where the single chemicals did not induce effects. Similar results were found in another mixture study of three similarly acting anti-androgens, i.e. the AR antagonists flutamide, procymidone and vinclozolin (Metzdorff et al., 2007).

Table 8.1. Summary of effects found in dams and male rat pups exposed to epoxiconazole (EZ), mancozeb (MAN), prochloraz (PZ), tebuconazole (TZ) or procymidone (PRO), or a mixture of these pesticides (PMIX) from GD 7 to PND 16. Values are expressed as percent of control values for relative organ weights and as increase in number for nipple retention (control value = 0.0). Values for genital malformations of external male reproductive organs are shown as group mean of % offspring within each litter having score 1, 2 or 3 and the sum (control value = 0.0%). Statistically significant effects compared to controls are indicated by bold letters.

Endpoint	EZ 3.75 mg/kg	MAN 6.25 mg/kg	PZ 8.75 mg/kg	TZ 12.5 mg/kg	PRO 12.5 mg/kg	MIX 43.75 mg/kg	Dose-additivity?	Joint effect compared to effect of single chemicals at Mix-25 %
Gestation length, days	23.1	23.0	23.2	23.0	23.0	**23.6**	Yes, slight syner-gy at high doses	Marked joint effect; no significant effect of single chemicals
Nipple retention, PND 13, number	0.1	0.1	0.3	0.5	**2.8**	**5,3**	Yes, slight syner-gy at high doses	Marked joint effect; smaller significant effect of procymidone
Epididymis weight, PD 16, % of control	103	104	109	98	105	**90**	n.a	Joint effect; no significant effect of single chemicals
Prostate weight, PD 16, % of control	105	98	110	121	84	**68**	n.a.	Marked joint effect; no significant effect of single chemicals
Seminal vesicle weight, PD 16, % of control	93	110	84	103	75	**64**	n.a.	Marked joint effect; no significant effect of single chemicals
Prostate weight, adult offspring, % of control	108	85	100	100	85	**77**	n.a.	Joint effect; no significant effect of single chemicals
LABC[#] weight adult offspring, % of control	96	100	96	100	93	**89**	n.a	Joint effect; no significant effects of single chemicals
Genital malformations, % - score 1 - score 2 - score 3 - total	 3.6 0.0 0.0 3.6	 0.0 0.0 0.0 0.0	 0.0 2.8 0.0 2.8	 0.0 0.0 0.0 0.0	 0.0 0.0 0.0 0.0	 8.6 6.5 1.9 **17.0**	n.a	Marked joint effect; no significant effect of single chemicals

[#]: Levator ani/bulbocavernosus muscles; n.a: not analysed

In adult offspring, reduced absolute weight of LABC and prostate were seen in the highest mixture dose group compared to controls, but not in any of the groups exposed to the pesticides alone at the same dose levels (table 8.1). The prostate weight was also decreased at PN 16 and the persistence of the effect into adulthood demonstrates that this is a long-lasting effect. In the group exposed to the high dose of procymidone, prostate and liver weights were significantly reduced compared to controls.

Increased frequency and severity of genital malformation was found in the highest mixture group and the highest dose of procymidone. The percentage of offspring with genital malformations was markedly higher in the highest mixture group than in any of the groups dosed with the individual pesticides alone at the dose included in the mixture (table 8.1). The individual pesticides alone caused no statistically significant effect at this dose level. As such, these results show severe mixture effects at dose levels where the individual pesticides caused no effect when given alone.

Furthermore, pestimix exposure lowered sperm counts markedly in the highest dose group. Control values did not differ from historic controls and our finding therefore indicate that the lowered sperm counts are caused by developmental exposure to the mixture of endocrine disrupting pesticides. This is a very important and potentially quite alarming result in relation to the low sperm counts and declining sperm quality in humans reported during the last decades (Carlsen ***et al***., 1992, Jørgensen ***et al***., 2006). No effects on sperm motility parameters were seen in the exposed animals in the present study, which indicates that the pestimix affected the number but not the function of the sperm cells.

In the behavioural studies of motor activity levels no significant effects were seen in the mixture groups in either males or females. In the spatial learning test (the Morris maze) some significant effects of mixed pesticide exposure were seen. These effects were mainly seen when all 14 dose groups were analysed together, but the group size for the single pesticide exposures was very small in regard to assessment of behavioural results (n=2-6). Therefore, the emphasis is rather put on the results from the statistical analysis where the number of litters was around 10 (i.e. analysis where only the control group and the three mixture groups were included or analysis where the two doses of each single pesticide were combined to represent one dose group). In these analyses, the only statistically significant effect indicating a mixture effect was seen on male swim latency on day 7, as the high mixture group showed an increased latency to reach the platform compared to controls, while no statistically significant effects were seen in males exposed to the single pestcides. As day 7 is the last day of the learning period, this finding could indicate a mixture effect on male learning. Males generally perform better that females in spatial learning tests and this was also seen in this study. Therefore the possible impaired learning in the male but not the female offspring could be due to endocrine disruption affecting the sexual differentiation of the brain. More studies are however needed before clear conclusions can be drawn.

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

Since at least one (mancozeb) and possibly more of the tested pesticides (the azole pesticides) may affect the thyroid hormone system, several endpoints used to investigate thyroid disruption were included in the study. Pup body weight and assessment of the behavioural endpoints are sensitive endpoints for discovering the effects of thyroidal disruption during development. Furthermore, weight and histopathology of the thyroid and levels of thyroid hormones in blood provide a direct measure of thyroidal disruption.

On pup day 16, weights of thyroids were not significantly different between groups, and neither were the thyroid weights in adulthood. Thyroid histology (control and all three mixture groups) was evaluated in adult female offspring, and no clear differences between groups were observed. The activity of the thyroid was generally not very marked in the evaluated histological sections. Follicles with high columnar vacuolated epithelial cells indicating higher degree of activity were only observed in few animals, mainly belonging to group 8 (high dose mancozeb). Correspondingly the number of animals with follicles dominated by columnar to cuboidal epithelium were significantly higher in group 8 compared to the control group. Mancozeb has previously been shown to produce structural and functional changes in thyroid of rats including hyperplasia and hypertrophy of follicular cells (Trivedi et al., 1997, Kackar et al., 1997).

T4 levels were measured in male and female pups at PD50 in control and mixture groups, and here no significant changes were seen in any dose group. No effects on pup body weights was seen in the mixture groups, or in the animals from any of the single pesticide exposure groups including mancozeb and also no changes in the learning and memory of the animals was observed in the Mancozeb groups. In the activity test a significantly elevated activity level was seen in the high dose Mancozeb females. This could be a real finding but other studies in our laboratory using a larger group sixe have not found this (Axelstad et al., 2011). Consequently, this finding may also be a false-positive result due to the relatively low number of offspring in the mancozeb group. All in all, no significant changes indicating anti-thyroid properties of the pestimix were observed.

8.2 Modelling of the mixture effects

The second objective of this project was to investigate if modelling of the expected mixture effects based on data for the single chemicals and using dose-addition or independent action could give useful estimates of the observed mixture effects for relevant endpoints.

Modelling was not done for the reproductive organ weights and the genital malformations, because there were insufficient data for the single pesticides regarding these endpoints.

For the endpoint gestation length dose-addition prediction at low doses gave a good prediction of the observed mixture effects. Both dose-additivity and independent action underestimated the mixture effects at high doses. However, the underestimation was generally smaller for the dose-additivity prediction than the independent action prediction. As there are no earlier studies of mixture effects on gestation length, our results showing that such effects can be predicted using dose-additivity provide new important knowledge

A similar picture was seen for the anti-androgenic endpoint nipple retention. In male offspring, the prediction of the mixture effect based on dose-additivity was in agreement with the observed effects at low doses, as there were no statistically significant differences between the predicted and the observed effect doses. However, dose-additivity underestimated the effects for the high doses of the mixture. The independent actions predictions were seen to even stronger underestimate the effects for high doses, and overestimate the effect for the low doses. That dose-additivity gives a good prediction of mixture effects on nipple retention has been found earlier after exposure to other mixtures of similarly or dissimilarly acting anti-androgens in several rat studies both by us and others (Hass et al., 2007 Rider et al., 2008, Christiansen et al., 2009).

For the endpoint anogenital distance index (AGDI) in females, both independent action and dose-additivity predictions highly underestimated the effect of the pesticide mixture.
So, although no statistically significant effect was seen in the combined analysis of the present studies and historical data for epiconazole, mancozeb, procymidone and tebuconazole individually, some of them seem to have contributed to the effect in the mixture. The present studies actually showed significant effects of the low doses of prochloraz, tebuconazole and epoxiconazole, whereas no effect on female AGDI has been seen in the present studies or our historical data for procymidone and mancozeb. Also, earlier studies in our laboratory have shown similar effects of prochloraz, epoxiconazole and tebuconazole (Laier et al., 2006; Taxvig et al., 2007). The effect in the mixture groups is therefore most likely caused by the combined exposure to the three azole fungicides. As there are no earlier studies of mixture effects on this endpoint and our results are limited further studies in this area appear warranted.

8.3 Chemical analysis of pesticides in blood

The third objective was to investigate the blood levels of the pesticides in rat dams and offspring, in order to evaluate whether the exposure to the mixture had caused higher blood levels of the pesticides than exposure to the single pesticides alone.

Procymidone, epoxiconazole, tebuconazole and prochloraz could be detected in all serum samples from dams. Procymidone occurred at highest levels. Epoxiconazole and tebuconazole resulted in similar levels when administrated as part of the mixture, even though the dose of epoxiconazole in the mixture was lower than for tebuconazole. The transfer of epoxiconazole, to the pups, was indicated to be more efficient than for tebuconazole. Also by single compound exposure the serum levels of epoxiconazole were found to be higher than for tebuconazole, even though the dose of epoxiconazole was lower than for tebuconazole. Prochloraz was detected in some pup serum samples, but all levels were below LOD.

The serum levels in the dams were as expected generally higher than those in the pups exposed indirectly via maternal milk. The best data were available for procymidone and epoxiconazole. The results, based on group means, showed that the serum level in the pups was around 10% of maternal level for procymidone, whereas it was only around 0.6% for epoxiconazole. The comparison was also done based on individual litters in the cases where there were measures for both dams and pups. The levels in the pups ranged from

8.2-15.5% of the levels in their dams for procymidone and from 0.2-0.9% for epoxiconazole.

By comparing the results obtained from the analysis of the pup serum samples from the mixture groups in range-finding study 2 on PD 13 with those from the PD 16 pups exposed to the pesticides alone in the extended mixture study, mixture effects are indicated for procymidone and epoxiconazole (table 8.2). The same dose resulted in more than two fold higher internal doses in the pups when the pesticides were administrated as part of the mixture than when administrated alone. The most likely explanantion for this finding is that the pesticides are interacting with respect to toxicokinetic issues like absorption, distribution, metabolism and/or excretion. Among these hypotheses the most likely one is that a slower excretion after mixture exposure occurred due to overload of the metabolic pathways. The metabolic capacity of pups develops gradually before and after birth. The higher internal doses after mixture exposure compared to single chemical exposure could therefore be due to the fact that the blood samples for the single chemical exposures were taken 3 days later than those for the mixture exposure. However, if the influence from this should be more than marginal, it would imply that major development in metabolic capacity should occur within these 3 days. We have no indications of that being the case and consequently the different time for the blood sampling is evaluated as likely to have only marginal influence on the difference seen between mixture and single chemical exposure. A more likely explanation is that slower elimination due to overload of metabolic pathways has occurred as several of the pesticides for example have similar types of phase II conjugates in adult animals. Prochloraz and to a minor extent procymidone are metabolized to phase II gluconoride conjugates and tebuconazole and prochloraz are metabolized to phase II sulphate conjugates (FAO 2004, EFSA 2009b, EC 2008b). For epoxiconazole, the most important phase II reaction consists of formation of glutathione adducts (EC 2008a). As none of the other pesticides are reported to form such adducts, the increased internal doses of epoxiconazole after mixture exposure does not appear likely to be due to overload of phase II metabolism. To clarify if metabolic overload after mixture exposure could be the explanation for the higher internal doses, detailed kinetics studies would be needed in developing rat pups and such are not available.

Table 8.2 Serum levels (ng/g serum) of procymidone and epoxiconazole in pups exposed to mixture (PD 13) or single pesticides (PD 16)

	Procymidon-dose, mg/kg bw/day	Procymidone serum level	Epoxiconazol-dose, mg/kg/day	Epoxiconazole, serum level
Pesticides given in mixture				
Median, positives	12,5	216,0	3,75	6,9
Median, positives	25	570,5	7,50	15,7
Pesticides given alone				
Median, positives	12,5	97,6	3,75	2,5
Median, positives	50	217,0	15,00	4,1
Ratio mixture/alone, similar doses		2.2		2.8
Ratio mixture/single, where single dose = 2 x mixture dose		2.6		3,8

The blood samples from both dams and pups were taken 1-5 hours after exposure of the dams in the morning. Ideally, the blood samples should have been taken at several time points after the dosing in order to find e.g. the peak levels. This was, however, not possible as the blood samples were taken when the animals were sacrificed. Alternatively, the blood samples could have been taken at the time when the peak was expected based on existing literature. Peak plasma concentration have for epoxiconazole been observed no later than 2 hours after administration to adult male and female rats (EC 2008a). For prochloraz, the plasma time course has shown a rapid initial absorption phase followed by a slower elimination phase and peak plasma levels in rats and dogs were found 8-24 hours after dosing (FAO 2004). For tebuconazole, the absorption from the gastro-intestinal tract of the rat is rapid and complete within 48 hours and peak levels were found from 20 to 100 min after administration (EC 2008b). Following single or multiple oral doses of procymidone, peak concentrations in tissues were reached 6-12 hours after dosing (EFSA 2009b). These data show that the approximate time when peak levels can be expected for the four pesticiedes varies considerably from around 1 to 24 hours. The pesticides were administered as a mixture and it was therefore not possible to take the blood samples at sacrifice at an optimal time for each of the pesticides in the mixture. Also, for the pups, the transfer via maternal milk depends on when the pups have suckled in relation to the time interval between dosing and sacrifice. The relationship between the serum levels of procymidone and epoxiconazole in pups and the time of day for the blood sampling is shown in figure 8.1. No clear relationship is seen indicating that the time for the blood samling has not had any major effect on the results.

The measures of serum levels included only the parent compound. All of the pesticides are metabolised to a large number of biotranformation products. Optimally, the serum levels of metabolites should also have been analyzed. This was, however, not possible due to lack of analytical methods for the many metabolites and especially the limited amount of blood available from the pups. This is not considered as a major limitation as the parent compounds based on our ***in vitro*** results are expected to cause the endocrine disrupting effects.

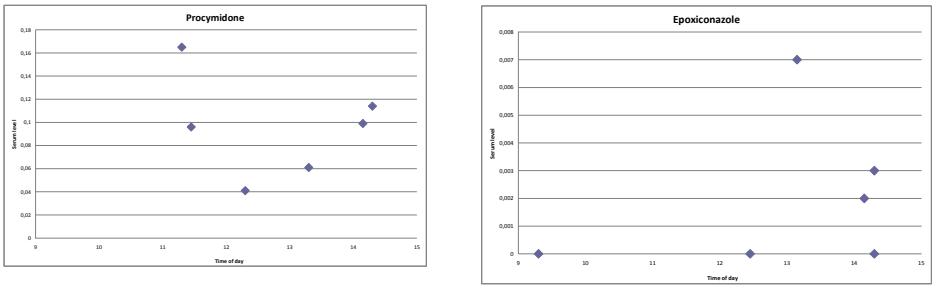


Figure 8.1 Extended mixture study. Serum levels (ng/g serum) of procymidone (left) and epoxiconazole (right) in pups and time of day for sampling. The dosing of the dams took place between 8 and 9 am.

8.4 *In vitro* studies

The objective of performing the ***in vitro*** assays was to evaluate the predictability of ***in vitro*** methods for estimating ***in vivo*** developmental effects.

The ranking of the potencies for AR antagonism was: Mixture ~ Procymidone > Prochloraz ~ Epoxiconazole > Tebuconazole. As the content of procymidone in the mixture actually constitutes only approximately 1/3 of the total mixture dose, these results indicate combination effects on this endpoint. This is in line with previous observations that chemicals act additively to antagonize AR (Birkhøj, 2004)

Prochloraz, tebuconazole, epoxiconazole and the mixture was shown to reduce the testosterone level in the cells, whereas procymidone and mancozeb had no effect.
Prochloraz, epoxiconazole, tebuconazole and the mixture also reduced estradiol levels, whereas procymidone and mancozeb at higher concentrations above 10 µM caused increased estradiol levels. The azole compounds and the mixture caused an increase in progesterone levels whereas the remaining chemicals were without any detectable effect. In general the effects of prochloraz was most pronounced on all three hormones.

The question is whether the ***in vitro*** results can be translated to ***in vivo*** effects in developmental rat studies. Overall, mancozeb did not have much effect in the ***in vitro*** assays. besides a general cytotoxic effect. This lack of effect fits well with the lack of effects on the studied endpoints in the rat studies.
In relation to the ***in vitro*** results from the H295R assay, if these results were to be used as an indicator for what we could expect to see in the ***in vivo*** experiments, one might have expected to seen an effect on the measured hormone levels, particularly on the progesterone levels in the dams. However, our experience from previous ***in vivo*** studies with azole fungicides is that effects on the hormone levels is mainly seen around the time of birth, e.g. on GD 21. So with that in mind, it is not so surprising that we do not find an effect on the measures hormone levels in the current studies, as the measurements have been performed at later time points.

Concerning the prediction of anti-androgenic effect (e.g. nipple retention), we would expect from the ***in vitro*** studies that procimidone and the 3 azole fungicides had the potential to induce this effect. Procymidone was the most potent AR antagonist ***in vitro*** and also had effects on nipple retention. Prochloraz was the most potent testosterone inhibiting chemical ***in vitro*** and was found to affect nipple retention in this study and has previously been shown to reduce male AGD ***in vivo*** although higher doses than those used in this project are necessary to induce this effect. All 3 azoles are able to induce progesterone levels ***in vitro*** (Kjaerstad et al., 2010) and this is this most likely the cause of the increased gestation length ***in vivo*** as a decline in progesterone levels in the dams is a prerequisite for on-time delivery in rats (Christenson and Devoto, 2003). The progesterone has to be measured in the dams around delivery which was not done in this project. In a previous study pregnant rats were dosed with tebuconazole (50 and 10 mg/kg bw/day) and epoxiconazole (15 and 50 mg/kg bw/day) from GD 7 until PD 16 and we found an increased gestational length as well as a marked increase in progesterone levels in plasma from both tebuconazole- and epoxiconazole-exposed dams at GD 21 (Taxvig et al., 2007).

All taken together, the ***in vitro*** studies gave some very good indications of whether to expect ***in vivo*** anti-androgenic effects and effect on gestation length.

8.5 Survey of pesticide intake and risk

The aim was to estimate the cumulative exposure from a group of pesticides with dissimilar mode of action but with the same effect. The exposure to multiple compounds with common effects present in more than one food simultaneously can be estimated by applying the relative potency factor (RPF) approach and by using the probabilistic approach. The RPF approach assumes that the effects following cumulative exposure can be predicted by dose-addition. The advantages by using the probabilistic method are that the whole distribution of residues in foods and the whole distribution of consumption data are combined in the calculations to give a distribution of all possible intakes. The variation in the populations eating habits are taken into account meaning that the individual bodyweights are used in the calculations. Finally the uncertainties at different percentiles are quantified.

Intake calculations were performed for different consumer groups; children, women and the whole population. Since the endocrine disrupting effect on nipple retention or increased gestation length are of interest for women in the childbearing age the intake calculations were performed for women in the age of 15-50 years old. The intake calculations have also been performed for the general population in the age 4-74 years and to children in the age 4-6 years. Even though it is well known that women eat more fruit and vegetables than men, the intake calculation for the women was a little lower than for the general population. This was caused by the fact that children eat relatively more per kg body weight compared to adults and therefore their intake was double as high as for the general population. So the children had a high impact to the exposure calculated for the general population.

Which percentile should be used as threshold of concern in the probabilistic exposure calculation depends on what risk the risk manager is willing to accept. In the US EPA (US EPA 2000) the percentile 99.9 is used as reference point. This percentile was also used in this study to evaluate the risk for the consumer.

Which pesticides contributed most to the intake? For the endpoint ‘nipple retention’ four of the pesticides (procymidone, tebuconazole, prochloraz and epoxiconazole) contributed to the effect in animal studies. Procymidone contributed far most to the cumulative exposure compared to the pesticides tebuconazole, epoxiconazole and prochloraz. Procymidone is found in many different high consumption commodities, but procymidone is not allowed to use in EU any longer, so in the future the residue content should not be found in commodities produced in the EU. However, commodities produced outside EU may still have residue content. The contribution to the exposure from prochloraz was low compared to procymidone. It is the intention that this substance will be withdrawn from Annex I to Directive 91/414 EEC 12/2011 with a period of grace until 12/2012. However an application has been resubmitted for inclusion. So at this stage it is unsure if prochloraz still will be found in commodities produced in the EU. Tebuconazole and epoxiconazole are included in Annex I to Directive 91/414 EEC. Tebuconazole is used in a huge amount of commodities in the EU. The substance is however not so potent with regard to endocrine disrupting properties and the contribution to the human exposure is relatively low. Epoxiconazole was only found in one commodity, which was leek. The potency for nipple retention was low compared to prochloraz, so the contribution to the risk was therefore also low.

For the endpoint 'gestation length' only the three substances prochloraz, epoxiconazole and tebuconazole contributed to the observed effect. Epoxiconazole had the highest potency but due to only one positive sample this substance did not contribute to the exposure to a great extent. Prochloraz was only found in citrus fruit, water melon and exotic fruit. Since the residue content is reduced by a factor of 10 by applying a processing factor (due to non-consumable peel) to the contents in citrus fruits and water melon, this substance did not contribute to a great extent to the exposure. Tebuconazole is used and found in many commodities but due to the relatively low potency compared to prochloraz this substance does not contribute to a great extent.

In the present study processing factors were used in the calculations for citrus fruits and water melon. Processing factors were not used for exotic fruits despite that many of these fruits have a peel that is not consumed. So if a processing factor had been applied for those exotic fruits with peel the estimated exposure would have been lower. So the calculations represent a worst case scenario of the exposure. However, the DNEL of 25 µg/kg bw/day for both nipple retention and increased gestation length was not exceeded at the 99.9 percentile.
Due to many analyses in citrus fruits and application of a processing factor of 0.1 to these values the impact of using ½ level of reporting instead of zero in the calculations did not have a great impact on the estimated exposure compared to zero. As described by Raaij et al. (2005) it is probably closer to reality assuming that all non-detects are true zeros than setting all non-detects at ½ Level of reporting.

To summarize, despite the uncertainties in the calculations of the dietary cumulative intake, the results indicated that the cumulative intake for Danish consumers is below 100 % of the DNEL of 25 µg/kg bw/day for prochloraz, for both on nipple retention and increased gestation length at the 99.9 percentile for women in childbearing age. A potential limitation is, however, that there were no Danish intake data for epoxiconazole, which is potent for inducing effect on gestation length. Therefore, data from Sweden were used and these data may be limited as there was only one positive sample from leek.

The calculation of the DNEL for the index compound prochloraz included an additional uncertainty factor of 3 to allow room for human exposure to other endocrine disrupting chemicals than the pesticides in the studied mixture. For effects on nipple retention and male sexual development in general, the relevant chemicals would include for example the anti-androgenic phthalates (DEHP, DBP etc) and other anti-androgenic pesticides such as vinclozolin and linuron. We have in another project, estimated the contribution from these chemicals to potential mixture effects based on estimated human exposure levels and the LOAELs for anti-androgenic effects. The results of this estimation support that the uncertainty factor of 3 should be sufficient (Christiansen et al., 2011 (submitted)). For effects on gestation length, the relevant chemicals would include a number of other imidazole pesticides and potentially also other chemicals with similar mode of action. As there is limited knowledge concerning this effect and mode of action, it cannot be evaluated whether the additional uncertainty factor of 3 for exposure to other similarly acting chemicals is sufficient for covering such chemicals. In addition, thousands of chemicals may potentially be endocrine disrupters based on ***in vitro*** studies and QSAR modelling, but ***in vivo*** data are scarce (Jensen et al., 2011, Vinggaard et al., 2008).

8.6 Overall discussion

The present project has shown mixture effects of endocrine disrupting pesticides and as such support that there is a need for cumulative risk assessment. Below the mixture results are discussed in relation to values used in regulatory risk assessment of single chemicals such as LOAELs, BenchMark Doses (BMDs), Derived No Effect Levels (DNELs) and Acceptable Daily Intake (ADI).

For the mixture effects on gestation length and nipple retention, we compared the calculated BMDs (effect doses in chapter 4) for these two endpoints to the LOAELs for the pesticides singly and in the mixture. The main purpose was to evaluate whether the mixture exposure leads to lower LOAELs or BMDs than those after single chemical exposure. BMDs are alternatives to the use of NOAEL and LOAEL in the risk assessment. Consequently, BMDs were also compared to LOAELs to evaluate which values are most protective.

Table 8.3 shows the LOAELs and BMDs for effects on gestation length. Mancozeb and procymidone are not included as these pesticides did not affect gestation length. All BMD values for mixture exposure to the pesticides were 3-10 times lower that the respective BMDs for single chemical exposure, and mixture LOAELs were approximately 6 times lower than the single chemical LOAELs. This is an important finding as it illustrates that mixture exposure leads to markedly lower effect levels.

For the single chemical exposure the LOAELs for epoxiconazole and tebuconazole lie between BMD10 and BMD25, whereas for prochloraz the LOAEL is higher than BMD25. For the mixture exposure, the LOAELs for all 3 pesticides lie between BMD10 and BMD25. This indicates that BMD10 is a more protective effect level than a LOAEL, whereas BMD25 is less protective than a LOAEL.

Table 8.3 Gestation length, LOAELs and Bench mark doses (BMD) after exposure to the pesticides alone or in mixture.

Pesticide	Epoxiconazole		Prochloraz		Tebuconazole	
	single	mixture	single	mixture	single	mixture
LOAEL	15.0	2.5	>35	5.8	>50	8.3
BMD10	6,2	1.5	15.3	3.6	48.3	5.1
BMD25	37.6	4.4	30.4	9.3	79.7	13.3

Mancozeb and procymidone are not included as these pesticides did not affect gestation length.

Table 8.4 shows an overview of the LOAELs and BMDs for effects on nipple retention in male offspring after exposure to the pesticides alone or in the mixture. Mancozeb is not included as this pesticide did not affect nipple retention.
As seen for gestation length, several of the values for mixture exposure to the pesticides were markedly lower than those for single chemical exposure. However, for procymidone, there was no difference between single and mixture LOAELs and BMD10. Also, the differences between single and mixture LOAEL for prochloraz and tebuconazole was only 2 times, and not 6 times like it was seen for gestation length. It must, however, be kept in mind that the mixture caused effect on nipple retention at the lowest mixture dose studied, i.e. the mixture LOAELs and BMDs may have been lower if lower doses of the mixture had been studied.

For the single chemical exposure, the LOAEL for epoxiconazole and procymidone lies between BMD10 and BMD25, whereas for prochloraz and tebuconazole the LOAEL is below BMD10. For the mixture exposure, the LOAELs for all 4 pesticides are between BMD10 and BMD25. This indicates that BMD10 is a more protective effect level than a LOAEL for epoxiconazole, procymidone and the mixture, but less protective for prochloraz and tebuconazole.

Table 8.4 Nipple retention in male offspring, LOAELs and Bench mark doses (BMD) after exposure to the single pesticides alone or in mixture.

Pesticide	Epoxiconazole		Prochloraz		Tebuconazole		Procymidone	
	single	mixture	single	mixture	single	mixture	single	mixture
LOAEL	>15.0	1.3	5.8	2.9	8.3	4.2	4.2	4.2
BMD10	12.5	0.2	14.9	0.5	13.8	0.7	0.8	0.7
BMD25	37.6	2.3	30.4	5.4	79.7	7.7	24.1	7.7

Mancozeb is not included as this pesticide did not affect nipple retention.

In table 8.5, the Acceptable Daily Intake (ADI) is compared to the DNEL for effect on nipple retention for each of the pesticides. The DNEL is calculated from the LOAELs and BMDs shown in tables 8.3 and 8.4 using the normally used uncertainty factors of 10 for extrapolation from animals to humans, 10 for different sensitivity among humans and 3 for extrapolation from an effect level to NOAEL. All of the DNELs based on LOAEL and BMDs for single chemical exposure to the pesticides are higher or similar to the ADI reflecting that the current ADI probably protects against the endocrine disrupting effect of the studied pesticides, when the pesticide is evaluated chemical by chemical. The mixture DNELs are, however, in most cases lower than the ADIs. This is especially the case for epoxiconazole and tebuconazole where the DNELs based on the respective LOAELs are half of the ADI and the DNELs based on BMD10 are 8 to 15 times lower than the ADI. This indicates that the ADIs are not sufficiently low to protect against the mixture effects of the pesticides shown in this project. An exception is procymidone where the ADI is lower than or similar to all of the mixture DNELs. Hhowever, as earlier mentioned, the mixture DNELs may actually be lower than the ones shown, because the mixture caused effect on nipple retention at the lowest mixture dose studied and may cause effects at even lower mixture doses.

Table 8.5 ADI and Derived No Effect Level (DNEL) in mg/kg bw/day for nipple retention in male offspring after exposure to the single pesticides alone or in mixture. DNEL is calculated as LOAEL or BMD divided by 300. Mancozeb is not included as this pesticide did not affect nipple retention.

Pesticide	Epoxiconazole		Prochloraz		Tebuconazole		Procymidone	
ADI*	0.008		0.010		0.030		0.003	
	single	mixture	single	mixture	single	mixture	single	mixture
DNELs based on:								
LOAEL	0.050	0.004	0.019	0.019	0.028	0.014	0.014	0.014
BMD10	0.042	0.001	0.050	0.002	0.046	0.002	0.003	0.002
BMD25	0.125	0.008	0.101	0.018	0.266	0.026	0.080	0.026

*The ADI values for the four pesticides respectively are from EC 2008a, FAO 2004, EC2008b and EFSA 2009

9 Conclusions

This project generally aimed at exploring the hypothesis that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides may lead to adverse developmental toxicity effects.

The extended mixture study as well as the range-finding studies showed clearly that a mixture of environmentally relevant endocrine disrupting pesticides, i.e. epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, caused adverse developmental toxicity effects, including long-term delayed effects, at dose levels below NOAELs for the individual pesticides. The mixture effects in the offspring were mainly seen in the males and included increased nipple retention, decreased weight of reproductive organs (epididymis, prostate and seminal vesicles), increased frequency of genital malformations and decreased sperm count. Prostate weight was decreased both in pups and in adult offspring showing that this was a long term effect. Weight of the levator ani/bulbocavernosus muscles (LABC) were decreased only in the adult male offspring, indicating long-term delayed mixture effects. All of these effects can typically be seen as results of exposure to anti-androgens during male sexual development and the mixture effects are therefore most likely caused by the combined exposure to epoxiconazole, prochloraz, tebuconazole and procymidone. Similar mixture effects has to some extent been shown earlier after exposure to other mixtures of anti-androgens.

The extended mixture study as well as the range-finding studies also clearly showed mixture effects at dose levels below NOAELs for the individual pesticides on gestation length and the ability of the dams to give birth. These are new findings and the mixture effect on the dams is most likely due to the combined exposure to epoxiconazole, prochloraz and tebuconazole.

The modelling of the expected mixture effects based on data for the single chemicals showed that dose-addition gave a good prediction of the observed mixture effects for the endpoints nipple retention and gestation length at lower dose levels. At higher dose levels, dose-addition underestimated the mixture effects. This could be related to toxicokinetic differences between single chemical and mixture exposure as investigations of the blood levels of the pesticides in rat offspring indicated that the mixture exposure caused higher blood levels than exposure to the single pesticides alone.

The same mixture of pesticides was studied using ***in vitro*** assays. All pesticides except for mancozeb and the mixture exhibited AR antagonism ***in vitro***. The ranking of the potencies for AR antagonism was: Mixture ≈ Procymidone > Prochloraz ≈ Epoxiconazole > Tebuconazole. As the content of procymidone in the mixture constituted only approximately 1/3 of the total mixture dose, these results indicated combination effects on AR antagonism ***in vitro***. In the H295 steroid synthesis assay prochloraz, tebuconazole, epoxiconazole and the mixture was shown to reduce testosterone and estradiol levels in the cells. In general the effects of prochloraz was most pronounced on all the hormones. The overall conclusion on the ***in vitro*** data is that the AR

reporter gene assay and the H295R steroidogenesis assay give some very good indications whether a chemical will induce effects ***in vivo***. In general dose-additivity is found in these assays, which is in line with most of the ***in vivo*** predictions.

The probabilistic survey of the dietary cumulative intake of the studied pesticides indicated that the cumulative intake for Danish consumers is below 100 % of the DNEL of 25 µg/kg bw/day for prochloraz at the 99.9 percentile for women of childbearing age. Despite the uncertainties in the calculations of the dietary cumulative intake, this result did not show reason for concern in relation to mixed exposure of Danish consumers to the investigated pesticides. A potential limitation is, however, that there were no Danish intake data for epoxiconazole, which is potent for inducing effect on gestation length, so limited data from Sweden were used. Calculation of DNEL for the index compound prochloraz included an additional uncertainty factor of 3 to allow room for human exposure to other endocrine disrupting chemicals than the pesticides in the studied mixture. For effects on nipple retention and male sexual development in general, an estimation of the exposure to other known anti-androgenic chemicals supports that the uncertainty factor of 3 should be sufficient. For effects on gestation length, it cannot be evaluated whether the additional uncertainty factor of 3 for exposure to other similarly acting chemicals is sufficient, due to lack of data. In addition, thousands of chemicals are potential endocrine disrupters based on ***in vitro*** studies and QSAR modelling (Jensen et al., 2011, Vinggaard et al., 2008).

Comparisons of LOAELs and BMDs for mixture effects on gestation length to LOAELs and BMDs from single chemical exposure showed that all mixture LOAELs were approximately 6 times lower than single chemical LOAELs. This is an important finding as it illustrates that mixture exposure leads to markedly lower effect levels. For nipple retention, several of the values for mixture exposure to the pesticides were also markedly lower than those for single chemical exposure. However, for procymidone, there was no difference between single and mixture LOAELs. Furthermore, the mixture caused effect on nipple retention at the lowest mixture dose studied, i.e. the mixture LOAELs may have been lower if even lower doses of the mixture had been studied.

Comparisons of the Acceptable daily intake (ADI) to the DNEL for effect on nipple retention based on LOAEL and BMDs for single chemical exposure to the pesticides showed that all DNELs were higher or similar to the ADI. This indicates that the current ADI should protect against the endocrine disrupting effect of the studied pesticides, when the pesticide is evaluated in isolation. The mixture DNELs were, however, in most cases lower than the ADIs. This is especially the case for epoxiconazole and tebuconazole where the DNELs based on the respective LOAELs were half of the ADI. This indicates that the ADIs are not sufficiently low to protect against the mixture effects of the pesticides shown in this project. Also, as earlier mentioned, the DNELs for the single pesticides in the mixture may actually be lower than the ones shown, because the mixture caused effect on nipple retention at the lowest mixture dose studied.

In summary, the present project has shown that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides caused adverse effects on male sexual development and gestation length in the dams. Investigations of the blood levels of the

pesticides in rat offspring indicated that the mixture exposure caused higher blood levels than exposure to the single pesticides alone. The mixture effects on gestation length and nipple retention were well predicted by dose-addition at low doses. The ***in vitro*** data showed that the AR reporter gene assay and the H295R steroidogenesis assay gave good indications for the observed ***in vivo*** effects. The probabilistic survey of the dietary cumulative intake did not show a reason for concern in relation to mixed exposure of Danish consumers to the investigated pesticides. However, it is somewhat uncertain, whether the additional uncertainty factor of 3 used to allow room for human exposure to other endocrine disrupting chemicals was sufficient for covering the already known endocrine disrupters and especially the thousands of chemicals that are potential endocrine disrupters based on ***in vitro*** data and QSAR modelling. Comparisons of the ADIs to the mixture DNELs for effect on nipple retention and gestation length indicate that the ADIs are not sufficiently low to protect against mixture effects. Thus, the results of the projects implies that risk assessment based on NOAELs for single chemicals can underestimate the risk and that there is a need for modification of risk assessment procedures for pesticides in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction.

10 Perspectives

10.1 Research perspectives

The present project has shown that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides caused adverse effects on male sexual development and gestation length. The mixture effects on gestation length and a sensitive endpoint for effects on male sexual development, i.e. nipple retention were well predicted by dose-addition at low doses. For effects on male sexual development our new findings confirm previous studies from both our laboratory and others showing that mixture effects of dissimilarly acting anti-androgens can be predicted by dose-addition. For mixture effect on gestation length, there are no previous data and further research in this area would therefore be relevant. For example, mixture studies of other chemicals affecting gestation length than the pesticides studied by us would be useful.

The extended mixture study also indicated that mixed exposure to endocrine disrupters can lead to increased female AGD. This is a new finding and more research of both single chemicals and mixtures are needed in this area.

The prediction based on dose-additivity under-predicted the mixture effects at high dose levels. This may be related to limitations in the data for the effects of the single pesticides. This is the case for epoxiconazole and to some extent also for tebuconazole and further studies of dose-response relationship for especially the relatively potent epoxiconazole would be useful for both the pesticide as such and for later predictions of mixture effects. In general, good dose-response data are need for predictions of mixture effects. Pesticides, including epoxiconazole, are however investigated using the 2-generation guideline study (OECD TG 416) before approval for use and this guideline does not include a number of sensitive endpoints for effects on male sexual development, e.g. nipple retention. This means that pesticides may show endocrine disrupting effects in spite of negative results in the 2-generation study. Thus there is a need for including sensitive endpoints in future studies, so the potential of pesticides for inducing endocrine disruption alone can be evaluated and the data can be used for predictions of mixture effects. Investigations of the blood levels of the pesticides in rat offspring indicated that the mixture exposure caused higher blood levels of procymidone and epoxiconazole than exposure to each pesticide alone. It was not possible to evaluate whether or not this was also the case for the other pesticides. Further studies need to be performed using more sensitive methods. To clarify if metabolic overload after mixture exposure could be the explanation for the higher internal doses, detailed kinetics studies would be needed in developing rat pups.

The ***in vitro*** data showed that the AR reporter gene assay and the H295R steroidogenesis assay (OECD TG 456) gave good indications of the observed ***in vivo*** effects. There are thousands of chemicals with no data about endocrine disrupting properties that humans may be exposed to. ***In vitro*** studies as well as QSAR modeling are much faster than animal studies and further studies using this methods are therefore very important for progress in

this area. Especially, such data are important for decisions on how to group endocrine disrupters for cumulative risk assessment.

The probabilistic survey of the dietary cumulative intake did not show a reason for concern in relation to mixed exposure of Danish consumers to the investigated pesticides. However, it is somewhat uncertain, whether the additional uncertainty factor of 3 used to allow room for human exposure to other endocrine disrupting chemicals was sufficient for covering the already known endocrine disrupters and especially the thousands of chemicals that are potential endocrine disrupters based on ***in vitro*** data and QSAR modelling. Further studies of effects of potential endocrine disrupters are therefore needed. Also, further exposure data and development of the methods for probabilistic survey of cumulative intake and exposure is highly relevant for risk assessment of mixture exposure

10.2 Regulatory perspectives

The present project has shown that combined developmental exposure to endocrine disrupting pesticides at dose levels below NOAELs for the single pesticides caused adverse effects on male sexual development and gestation length in the dams.
Thus, the results imply that risk assessment based on NOAELs for single chemicals can underestimate the risk and that there is a need for modification of risk assessment procedures for pesticides in order to take account of mixture effects and the potentially serious impact of mixed exposure on development and reproduction.

The mixture effects on gestation length and nipple retention were well predicted by dose-addition at low doses. This confirms previous studies from both our laboratory and others showing that mixture effects of dissimilarly acting anti-androgens can be predicted by dose-addition. Consequently, it is recommended to perform cumulative risk assessment based on dose-additivity unless there are data demonstrating that this is not the best method.

Pesticides are investigated using the current 2-generation guideline study (OECD TG 416) before approval for use, but this guideline does not include a number of sensitive endpoints for endocrine disrupting effects on male and female sexual development as well as effects on thyroid hormones. The newly accepted OECD Test, Extended One Generation Reproductive Toxicity Study (EOGRTS, TG 443) includes sensitive endpoints for endocrine disrupting effects on male and female sexual development as well as effects on thyroid hormones. In addition, developmental neurotoxicity is included to some extent and this gives a possibility for assessing if there are endocrine disrupting effects on sexual differentiation of the brain. The EOGRTS in contrast to the 2-generation study does not include mandatory mating of the 1st generation and production of 2nd generation offspring, but it is included as an option in special cases. Retrospective studies of around 500 2-generation studies have, however, indicated that the 2nd generation can be omitted without affecting risk assessment outcome or classification (Piersma et al., 2011, Rorije et al., 2011). Inclusion of the EOGRTS in to the EU Test Methods Regulation is currently being discussed. It is strongly recommended to use the EOGRTS instead of the 2-generation study in the future to avoid false-negative results or inappropriate NOAELs with respect to endocrine disruption and reproductive toxicity in general.

Investigations of the blood levels of the pesticides in rat offspring indicated that the mixture exposure caused higher blood levels than exposure to each pesticide alone. Consequently, internal blood levels of both parent compound and metabolites for both single chemicals and mixtures may provide a more relevant background for evaluating whether mixture effects can be predicted using e.g. dose-addition. This is therefore recommended if such data are available. The new EOGRTS (OECD TG 443) in contrast to the presently used 2-generation study includes optional studies of toxicokinetics for the chemical studied. It is recommended to have this option included in future studies as toxicokinetic data may be very useful in relation to later eventual mixture studies.

The ***in vitro*** data showed that the AR reporter gene assay and the H295R steroidogenesis assay (OECD TG 456) gave good indications for the observed ***in vivo*** effects in the present project. Also a number of other ***in vitro*** tests are useful for detection of endocrine disrupting properties, and for some of those, OECD guidelines are available or on the way. Knowledge of mode of action is important for grouping of chemicals for cumulative risk assessment. Thus it is recommended that chemicals with ***in vivo*** indications of endocrine disrupting effects, but where data are too uncertain to conclude on the mode of action, are studied further using ***in vitro*** tests.

Also, it is recommended that the grouping of chemicals for cumulative risk assessment is based on ***in vitro*** data, QSAR results and similar types of effects in ***in vivo*** studies. Concerning the latter, e.g. decreased AGD and increased nipple retention in male offspring signal anti-androgenic effects.

The probabilistic survey of the dietary cumulative intake did not show a reason for concern in relation to mixed exposure of Danish consumers to the investigated pesticides. However, these results are not sufficient for concluding no concern for mixture effects, as humans may be exposed to many not yet identified endocrine disrupting chemicals.

Comparisons of ADIs to mixture DNELs for effect on nipple retention and gestation length indicated that the ADIs are not sufficiently low to protect against mixture effects. It is not proposed to lower the ADIs in order to cover potential mixture effects, but instead it is proposed to consider mixture effects when establishing e.g. Maximum Residue Values (MRLs) for pesticides. One possibility is to allow each pesticide to use only a fraction of the ADI, where the fraction should depend on the number of relevant endocrine disrupters in the specific exposure scenario. Another possibility is obviously to base the cumulative risk assessment on dose-additivity modeling.

Last but not least, it is recommended when considering cumulative risk assessment to include all types of chemicals, e.g. pesticides, industrial chemicals, drugs etc. as endocrine disrupters exist within all of these types of chemicals and humans may be exposed to several of them simultaneously.

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Appendix 1

Table 3.9. Extended Mixture Study. Pregnancy and litter data. Data represent group means based on litter means ± SD. Mix = pestimix, Epo= epoxiconazol, Mz=mancozeb, Prchl=prochloraz, Teb=tebuconazol, Prcy=procymidon, *** p < 0.001

Dams and litters	1: Control	2: Mix-14,58	3: Mix-29,17	4: Mix-43,75	5: Epo-3,75	6: Epo-15	7: Mz-6,25	8: Mz-25	9: Prchl-8,75	10: Prchl-35	11: Teb-12,5	12: Teb-50	13: Prcy-12,5	14: Prcy-50
No. of dams (viable litters)	15	17	10 (9)	15(14)	8	4	5	7	8	4	8	6	7	4
Maternal weight gain GD7-GD21	88.1 ± 13.2	88.6 ± 11.6	86.7 ± 8.6	77.1 ± 12.8	87.3 ± 16.1	84.0 ± 21.1	87.4 ± 5.7	84.1 ± 11.4	85.9 ± 9.6	85.0 ± 5.0	85.4 ± 15.6	82.5 ± 10.2	86.6 ± 10.0	85.0 ± 3.7
Maternal weight gain GD7-PD1	18.5 ± 9.8	17.4 ± 9.5	10.7 ± 8.3	14.6 ± 17.7	22.9 ± 10.2	20.8 ± 9.4	15.4 ± 7.3	13.3 ± 6.5	14.8 ± 4.8	8.8 ± 10.4	11.0 ± 9.6	17.3 ± 15.8	11.6 ± 8.2	6.3 ± 5.5
Body weight gain PD1-13	33.0 ± 9.5	35.5 ± 9.2	31.2 ± 9.3	22.9 ± 19.0	30.9 ± 9.7	33.5 ± 4.0	34.8 ± 8.6	37.3 ± 3.3	34.7 ± 6.1	36.0 ± 8.4	44.8 ± 9.2	33.7 ± 4.2	39.1 ± 6.5	41.8 ± 11.4
Gestation length (days)	23.0 ± 0.0	23.0 ± 0.0	23.5 ± 0.5***	23.6 ± 0.5***	23.1 ± 0.4	23.8 ± 0.5***	23.0 ± 0.0	23.0 ± 0.0	23.2 ± 0.4	22.8 ± 0.5	23.0 ± 0.0	23.1 ± 0.2	23.0 ± 0.0	23.0 ± 0.0
% postimplantation loss	11.0 ± 24.9	5.4 ± 6.9	6.4 ± 9.9	16.7 ± 25.9	11.7 ± 11.7	4.0 ± 4.6	0.0 ± 0.0	2.3 ± 4.0	3.7 ± 6.0	1.8 ± 3.6	7.9 ± 10.5	5.5 ± 6.5	13.4 ± 35.1	0.0 ± 0.0
% perinatal loss	14.9 ± 25.2	7.3 ± 8.6	23.4 ± 28.3	23.3 ± 28.6	13.2 ± 12.6	10.3 ± 15.8	1.8 ± 4.1	3.3 ± 4.2	5.4 ± 7.0	1.8 ± 3.6	8.6 ± 12.3	9.5 ± 5.7	17.3 ± 36.9	1.9 ± 3.8
Born alive per. litter	11.1 ± 2.3	11.9 ± 2.5	11.6 ± 2.4	9.8 ± 2.0	10.4 ± 1.8	8.8 ± 3.7	12.2 ± 1.2	12.9 ± 1.7	11.7 ± 1.8	12.0 ± 1.7	11,8 ± 1.9	11.5 ± 2.5	10.8 ± 3.0	12.8 ± 2.4
% Postnatal death	4.4 ± 9.4	2.1 ± 4.3	17.4 ± 30.8	8.9 ± 18.1	1.8 ± 5.1	6.8 ± 13.6	1.8 ± 4.1	1.0 ± 2.7	1.7 ± 5.1	0.0 ± 0.0	1.0 ± 2.9	4.1 ± 6.6	2.0 ± 5.4	1.9 ± 3.9
% Males	48.4 ± 18.8	47.4 ± 17.5	52.4 ± 13.5	51.6 ± 16.3	44.9 ± 28.1	36.0 ± 29.6	49.8 ± 13.7	45,4 ± 12.9	40.2 ± 11.9	54.3 ± 12.7	56.2 ± 15.2	47.4 ± 16.8	44.6 ± 19.6	53.6 ± 17.3

Table 3.10. Extended Mixture Study. Offspring data. Data represent group means based on litter means ± SD. Mix = pestimix, Epo= epoxiconazol, Mz=mancozeb, Prchl=prochloraz, Teb=tebuconazol, Prcy=procymidon, * p < 0.05, ** p < 0.01, *** p < 0.001

Offspring (data from viable litters)	1: Control	2: Mix-14,58	3: Mix-29,17	4: Mix-43,75	5: Epo-3,75	6: Epo-15	7: Mz-6,25	8: Mz-25	9: Prchl-8,75	10: Prchl-35	11: Teb-12,5	12: Teb-50	13: Prcy-12,5	14: Prcy-50
Male birth weight (g)	6.5 ± 0.4	6.1 ± 0.4	6.2 ± 0.4	6.4 ± 0.5	6.5 ± 0.2	6.6 ± 0.6	6.2 ± 0.1	6.2 ± 0.2	6.4 ± 0.4	6.3 ± 0.4	6.3 ± 0.4	6.1 ± 0.4	6.2 ± 0.3	6.4 ± 0.3
Female birth weight (g)	6.1 ± 0.4	5.9 ± 0.4	5.9 ± 0.5	6.1 ± 0.6	6.1 ± 0.3	6.3 ± 0.6	6.1 ± 0.2	5.9 ± 0.1	6.0 ± 0.4	6.0 ± 0.4	6.0 ± 0.3	5.9 ± 0.2	6.1 ± 0.2	6.1 ± 0.3
Male AGD (units)	24.5 ± 1.1	23.4 ± 1.2	24.1 ± 1.0	24.0 ± 1.2	25.7 ± 0.9 ***	23.9 ± 1.3	23.9 ± 1.0	24.0 ± 0.9	24.8 ± 0.6	24.6 ± 0.7	24.7 ± 1.3	24.6 ± 0.8	22.8 ± 0.9 **	22.0 ± 0.7 ***
Male AGD/cubic root bw (AGDI)	13.1 ± 0.5	12.8 ± 0.7	13.1 ± 0.5	12.9 ± 0.4	13.8 ± 0.4 *	12.7 ± 1.0	13.0 ± 0.6	13.1 ± 0.4	13.4 ± 0.4	13.4 ± 0.4	13.4 ± 0.7	13.5 ± 0.4	12.4 ± 0.4 ***	11.9 ± 0.3 ***
Female AGD (units)	13.6 ± 0.6	13.7 ± 0.8	14.1 ± 0.5	15.0 ± 0.8 ***	14.9 ± 1.0 **	14.3 ± 0.9	14.2 ± 0.9	13.5 ± 0.5	14.1 ± 0.8	14.5 ± 0.4 *	14.4 ± 1.0	14.5 ± 0.5 *	13.4 ± 0.4	13.3 ± 0.2
Fem. AGD/cubic root bw (AGDI)	7.4 ± 0.3	7.6 ± 0.5	7.8 ± 0.3 *	8.2 ± 0.3 ***	8.1 ± 0.6 **	7.8 ± 0.7	7.8 ± 0.5	7.5 ± 0.2	7.8 ± 0.5 *	8.0 ± 0.2 **	7.9 ± 0.6 *	8.0 ± 0.2 **	7.3 ± 0.2	7.3 ± 0.2
No. Areolas males ^	0.0 ± 0.0	1.9 ± 1.6***	3.3 ± 0.9***	5.3 ± 1.1***	0.1 ± 0.2	0.5 ± 1.0	0.1 ± 0.1	0.6 ± 0.6***	0.3 ± 0.2	1.7 ± 1.2***	0.5 ± 0.8	1.6 ± 0.4***	2.8 ± 1.2***	6.0 ± 2.0***
No. Areolas. females	12.0 ± 0.4	13.4 ± 3.9	12.4 ± 0.4	12.5 ± 0.4	12.4 ± 0.3	12.8 ± 0.5	12.2 ± 0.1	12.4 ± 0.1	12.3 ± 0.2	12.1 ± 0.2	12.5 ± 0.6	12.2 ± 0.2	12.2 ± 0.2	12.4 ± 0.4
Male body weight PD 6 (g)	12.9 ± 1.3	12.2 ± 1.3	12.0 ± 1.3	12.5 ± 1.5	13.1 ± 0.9	13.2 ± 1.0	12.3 ± 0.7	11.8 ± 0.4	12.7 ± 1.2	12.9 ± 1.7	12.1 ± 1.7	12.3 ± 1.2	12.4 ± 0.7	12.2 ± 1.4
Female body weight PD 6 (g)	12.5 ± 1.2	11.9 ± 1.4	11.6 ± 1.4	11.9 ± 1.3	12.8 ± 1.4	12.2 ± 1.0	12.1 ± 0.6	11.4 ± 0.5	12.1 ± 1.2	12.2 ± 1.7	11.9 ± 1.7	12.1 ± 1.2	12.2 ± 0.7	12.0 ± 1.1
Male body weight PD 13 (g)	26.1 ± 4.1	24.0 ± 2.7	25.1 ± 2.9	26.7 ± 3.9	25.9 ± 3.0	28.0 ± 2.4	24.6 ± 2.4	23.0 ± 1.0	25.6 ± 1.5	24.9 ± 2.7	24.2 ± 2.7	24.8 ± 2.0	24.9 ± 1.0	24.3 ± 2.9
Fem. body weight PD 13 (g)	25.4 ± 3.9	23.7 ± 3.0	24.4 ± 2.8	24.3 ± 4.6	26.0 ± 4.1	26.5 ± 2.2	24.4 ± 2.5	22.7 ± 1.4	24.6 ± 1.8	24.0 ± 2.7	23.9 ± 2.1	24.4 ± 2.4	24.5 ± 1.4	23.6 ± 2.8
Male body weight PD 22 (g)	45.6 ± 6.7	41.5 ± 4.4	44.0 ± 5.6	46.8 ± 7.0	43.3 ± 4.6	46.8 ± 3.5	42.9 ± 4.2	40.4 ± 1.6	43.2 ± 3.3	42.4 ± 5.0	43.0 ± 4.6	43.8 ± 4.5	42.5 ± 1.3	43.0 ± 4.4
Fem. body weight PD 22 (g)	45.2 ± 6.5	41.1 ± 4.2	41.9 ± 3.9	44.8 ± 5.8	46.3 ± 7.7	46.7 ± 3.2	42.6 ± 3.5	40.3 ± 2.2	42.3 ± 4.0	41.3 ± 4.9	42.7 ± 3.4	42.8 ± 4.5	42.4 ± 1.3	41.3 ± 4.9

^ The number of nipples in the control group males was zero and it was therefore necessary to put in 1 nipple in 3 pups from the control group to perform the statistical model.

Table 3.11 Extended Mixture Study. Female organ weights PD 16. No statistically significant differences between groups were detected for body weights and absolute or relative organ weights.

Absolute and relative organ weights, females	n	Body weight (g)	SD	Uterus (mg)	SD	Ovary (mg)	SD	Liver (mg)	SD	Uterus (mg/100 g bw)	SD	Ovary (mg/100 g bw)	SD	Liver (mg/100 g bw)	SD
1: Control	14	30,1	3,9	19,2	3,8	4,8	1,4	767	105	64,9	13,7	16,0	4,8	2565	151
2: Pestimix-14,58	16	27,6	2,9	17,8	3,8	5,0	1,6	717	99	62,4	8,9	17,9	5,4	2547	124
3: Pestimix-29,17	8	28,7	4,2	17,2	2,7	4,4	1,2	753	119	60,0	6,9	15,7	5,3	2610	123
4: Pestimix-43,75	11	29,3	3,7	18,8	2,8	4,9	1,0	783	128	62,9	7,9	16,4	3,3	2603	143
5: Epoxiconazol-3,75	8	31,1	4,5	17,9	1,8	4,7	0,7	797	123	59,1	8,5	15,5	3,8	2597	163
6: Epoxiconazol-15	4	32,7	4,4	20,2	0,9	5,0	2,2	789	123	66,3	9,6	16,3	6,8	2553	106
7: Mancozeb-6,25	5	27,8	3,0	18,5	2,1	5,1	1,1	747	118	64,8	11,5	17,5	2,9	2576	125
8: Mancozeb-25	7	26,5	2,5	17,2	2,7	4,7	1,0	680	62	64,9	11,5	17,8	3,4	2572	71
9: Prochloraz-8,75	9	29,4	2,8	18,9	1,8	4,8	0,5	757	63	64,5	5,8	16,5	2,3	2579	108
10: Prochloraz-35	3	29,9	2,5	18,6	2,7	5,2	1,0	796	61	62,1	7,9	17,2	3,1	2660	43
11: Tebuconazol-12,5	8	29,5	1,4	19,8	3,8	5,2	0,7	734	78	69,9	12,8	18,5	3,3	2586	118
12: Tebuconazol-50	5	30,7	2,0	19,0	2,4	5,0	0,7	776	66	64,0	6,3	16,9	1,8	2613	198
13: Procymidon-12,5	7	28,2	2,3	17,8	1,8	4,8	0,9	717	87	62,1	5,9	16,9	3,1	2500	190
14: Procymidon-50	4	27,0	2,4	15,6	0,2	4,2	0,7	744	141	55,5	6,6	14,7	1,6	2621	304

Tables 3.12 A and B Extended Mixture Study. Absolute and relative male organ weights PD 16. Statistically significant differences between controls and exposed are marked with asterisks which indicate significance levels: *indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001. P values result from ANCOVA using body weights as a covariate followed by Dunnett's test on all 14 groups (Absolute weights, Table A) and from ANOVA of relative weights followed by Dunnett's test on all 14 groups (relative weights, Table B). There are no statistically significant differences in body weight between controls and exposed groups. # indicates significantly different from controls in a model including only control and different doses of the same compound or the mixture using Dunnett's post hoc test. # p<0.05, ## p<0.01, ### p<0.001.
Ves.sem.: seminal vesicle; Bulbo: glandula bulbocavernosus; LABC: levator ani/bulbospongiosus muscle

Absolute organ weights (mg)	n	Body weight (g)	Testes	Epididymis	Prostate	Ves sem	LABC	Bulbo	Liver	Thyroid
1: Control	15	30.8±5.7	103±15	23.3±2.3	10.6±2.5	10.4±3.6	26.5±5.8	1.7±0.4	786±128	4.4±0.7
2: Pestimix-14.58	16	28.9±2.8	101±11	20.3±2.5*. ##	8.8±1.7	8.0±2.0	22.7±3.8	1.5±0.4	735±68	4.0±0.6
3: Pestimix-29.17	9	30.2±3.9	109±13#	20.8±2.2#	8.5±1.9#	8.6±1.8	26.4±6.7	1.5±0.4	811±120	4.4±0.8
4: Pestimix-43.75	12	30.7±4.5	110±15#	19.4±1.7 ***. ###	7.1±1.9***. ###	7.2±1.8**. ##	24.3±5.6	1.5±0.6	811±123	4.4±1.6
5: Epoxiconazole-3.75	6	30.9±4.5	106±7	22.3±1.6	10.9±1.7	9.5±1.0	26.1±15.5	2.2±0.8	789±135	4.8±1.4
6: Epoxiconazole-15	3	33.7±4.0	120±10	27.6±0. 9##	14.9±2.7*. #	9.8±3.0	25.1±3.5	2.2±0.6	902±87	4.3±1.0
7: Mancozeb-6.25	5	28.5±3.6	100±8	21.0±2.0	9.6±1.0	10.6±2.2	23.4±2.8	1.7±0.4	718±99	4.4±0.7
8: Mancozeb-25	7	27.2±1.1	97±7	22.6±1.5	9.8±3.1	8.6±2.2	25.7±4.7	1.7±0.3	690±38	4.3±0.9
9: Prochloraz-8.75	9	30.2±2.0	108±8	23.3±2.5	11.4±0.8	8.6±2.0	25.7±3.2	1.6±0.4	765±77	3.8±0.6
10: Prochloraz-35	4	30.0±3.5	107±19	20.1±2.6 #	10.8±2.4	7.8±1.8	22.8±2.5	1.5±0.5	760±130	3.4±0.4#
11: Tebuconazole-12.5	8	28.7±3.8	104±16	22.4±2.4	12.0±2.6	9.8±3.2	25.0±3.6	1.7±0.4	746±96	4.1±1.2
12: Tebuconazole-50	5	30.5±2.8	105±8	22.0±2.7	9.8±2.9	11.5±2.2	24.5±2.2	1.7±0.3	839±99##	5.3±1.2
13: Procymidone-12.5	6	29.8±1.0	112±7*,##	22.3±3.5	8.6±1.5	7.6±1.4	27.8±6.3	1.5±0.2	786±46	4.3±0.9
14: Procymidone-50	3	28.1±4.6	102±16	18.7±3.0#	6.5±0.7#	8.2±1.0	18.5±2.4	1.1±0.4#	771±194	4.2±0.6

Relative organ weights (mg/100g bw)	n	Testes	Epididymis	Prostate	Ves sem	LABC	Bulbo	Liver	Thyroid
1: Control	15	337±23	71.2±23.2	34.5±6.8	33.8±8.5	89.1±20.6	5.7±1.2	2557±114	14.5±2.8
2: Pestimix-14.58	16	350±38	70.7±9.3	30.4±5.4	27.9±6.3	79.2±10.7	5.1±1.4	2546±105	14.0±2.0
3: Pestimix-29.17	9	362±14	60.8±25.4	28.2±5.6	28.5±4.7	86.6±16.1	4.9±1.1	2677±68	14.8±2.6
4: Pestimix-43.75	12	357±13	63.7±6.5**. ##	23.4±5.9***. ###	21.5±7.9**. ##	82.4±16.0	5.0±2.0	2645±140	14.3±4.8
5: Epoxiconazole-3.75	6	348±34	73.2±8.8	36.1±7.3	31.3±6.5	98.7±19.5	7.3±2.9	2548±136	15.8±5.2
6: Epoxiconazole-15	3	358±38	82.8±11.3	44.8±11.7	28.6±5.3	74.6±7.4	6.6±2.1	2682±152	12.9±3.3
7: Mancozeb-6.25	5	351±17	74.4±8.4	33.9±4.1	37.1±5.3	82.2±5.1	5.9±0.8	2525±259	15.6±2.3
8: Mancozeb-25	7	359±29	71.9±32.1	36.0±11.3	31.7±7.9	94.5±16.0	6.2±1.1	2540±93	15.8±3.7
9: Prochloraz-8.75	9	357±23	77.4±10.2	38.0±3.5	28.4±5.9	85.4±12.1	5.3±1.3	2531±152	11.3±4.7
10: Prochloraz-35	4	355±36	67.1±5.2	36.1±6.6	26.7±9.6	76.1±5.0	5.0±1.8	2520±148	11.3±1.9
11: Tebuconazole-12.5	8	363±29	69.6±28.5	41.8±6.0	34.7±11.5	89.6±12.5	6.1±1.5	2599±70	14.2±3.3
12: Tebuconazole-50	5	346±19	72.4±8.4	32.3±9.6	37.7±6.6	80.6±6.4	5.4±0.8	2749±121##	17.2±3.1
13: Procymidone-12.5	6	376±22*, ##	74.8±11.2	28.8±5.1	25.3±4.5	93.3±21.0	5.1±0.5	2636±128	14.3±3.0
14: Procymidone-50	3	363±27	67.2±12.9	23.3±1.8	29.5±2.6	41.7±36.2	2.4±2.2#	2722±260	15.2±2.5

Table 3.16 Extended Mixture Study. Absolute and relative female organ weights, adult. No statistically significant differences between groups were observed for body weights or organ weights. Dunnett's test was performed comparing all 14 groups to controls. Furthermore, a model using different doses of the same compound or mixture using Dunnett's post hoc test was applied.

Absolute/relative organ weights. g	N	Body weight	Liver	Uterus	Ovary	Liver (g per 100 g)	Uterus(g per 100 g)	Ovary (g per 100 g)
1: Control	15	250±13	6.84±0.38	0.88±0.41	0.085±0.015	2.74±0.17	0.35±0.16	0.034±0.005
2: Pestimix-14.58	12	250±19	6.52±0.45	0.98±0.37	0.090±0.011	2.61±0.16	0.39±0.16	0.036±0.005
3: Pestimix-29.17	9	242±18	6.49±0.73	0.90±0.33	0.089±0.016	2.68±0.18	0.37±0.12	0.037±0.008
4: Pestimix-43.75	9	253±25	7.15±0.92	0.69±0.29	0.095±0.019	2.82±0.12	0.28±0.12	0.038±0.007
5: Epoxiconazol-3.75	6	266±17	7.08±0.37	1.07±0.22	0.092±0.015	2.66±0.06	0.40±0.10	0.035±0.005
6: Epoxiconazol-15	3	257±18	6.47±0.14	0.60±0.38	0.103±0.015	2.53±0.13	0.24±0.17	0.040±0.004
7: Mancozeb-6.25	6	248±15	6.42±0.46	0.75±0.28	0.083±0.012	2.59±0.09	0.30±0.11	0.034±0.005
8: Mancozeb-25	5	253±25	7.11±0.59	0.74±0.25	0.100±0.007	2.82±0.15	0.29±0.08	0.040±0.003
9: Prochloraz-8.75	8	253±28	6.83±0.67	0.90±0.34	0.090±0.020	2.71±0.20	0.35±0.12	0.036±0.007
10: Prochloraz-35	5	231±8	6.51±0.27	1.06±0.42	0.080±0.010	2.82±0.14	0.46±0.18	0.034±0.005
11: Tebuconazol-12.5	7	247±16	6.65±0.78	0.71±0.17	0.093±0.021	2.69±0.22	0.29±0.07	0.038±0.009
12: Tebuconazol-50	5	246±13	7.05±1.01	0.71±0.23	0.093±0.028	2.85±0.43	0.28±0.11	0.037±0.013
13: Procymidon-12.5	6	245±8	6.47±0.37	1.17±0.33	0.093±0.010	2.64±0.17	0.48±0.14	0.038±0.005
14: Procymidon-50	4	245±11	6.46±0.65	0.77±0.39	0.091±0.012	2.64±0.19	0.32±0.17	0.037±0.004

Table 3.17 A, B Extended Mixture Study. Absolute and relative male organ weights, adult. Statistically significant differences between controls and exposed are marked with asterisks which indicate significance levels: *indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001. P values result from ANCOVA using body weights as a covariate followed by Dunnett's test (Absolute weights, Table A) and from ANOVA of relative weights or body weight followed by Dunnett's test (relative weights, Table B). Dunnett's test was performed comparing all 14 groups to controls. # indicates significantly different from controls in a model including only control and different doses of the same compound or the mixture using Dunnett's post hoc test. # p<0.05, ## p<0.01, ### p<0.001.
Bulbo: glandula bulbocavernosus; LABC: levator ani/bulbospongiosus muscle

Abs. organ weights	N	Body weight	Testis	Epididymis	Prostate	Seminal vesicle	LABC	Bulbo	Liver	Thyroid
1: Control	16	497±34	3.89±0.31	0.70±0.07	0.63±0.15	1.91±0.37	1.38±0.19	0.22±0.07	13.2±1.4	0.022±0.003
2: Pestimix-14.58	18	458±29#	3.88±0.24	0.69±0.07	0.67±0.17	1.94±0.30	1.27±0.17	0.24±0.08	11.6±0.9	0.024±0.010
3: Pestimix-29.17	12	469±50	4.03±0.69	0.74±0.11	0.63±0.12	2.11±0.40	1.28±0.19	0.18±0.07	12.0±1.8	0.024±0.004
4: Pestimix-43.75	16	457±32	4.01±0.26	0.69±0.06	0.47±0.19#	2.08±0.38	1.15±0.23*. #	0.21±0.06	12.0±1.2	0.020±0.003
5: Epoxiconazol-3.75	10	472±41	4.00±0.17	0.66±0.07	0.63±0.14	1.94±0.35	1.24±0.17	0.21±0.05	11.9±0.6	0.020±0.002
6: Epoxiconazol-15	2	396±31#	4.06±0.03	0.75±0.001	0.64±0.13	2.09±0.001	1.28±0.08	0.29±0.06	11.4±0.1	0.023±0.001
7: Mancozeb-6.25	8	464±55	3.79±0.27	0.69±0.03	0.53±0.25	1.82±0.46	1.30±0.28	0.22±0.07	12.0±1.6	0.030±0.022
8: Mancozeb-25	10	478±36	3.89±0.34	0.72±0.08	0.61±0.14	1.90±0.43	1.23±0.09	0.21±0.07	12.9±1.1	0.029±0.015
9: Prochloraz-8.75	10	492±30	4.15±0.25	0.71±0.05	0.64±0.15	2.05±0.25	1.31±0.21	0.28±0.06	12.9±1.6	0.022±0.003
10: Prochloraz-35	6	437±18#	3.69±0.19	0.64±0.03	0.65±0.11	1.98±0.20	1.31±0.20	0.22±0.09	10.7±0.9	0.021±0.002
11: Tebuconazol-12.5	8	455±27#	3.84±0.41	0.69±0.12	0.59±0.13	1.80±0.29	1.24±0.16	0.17±0.06	11.3±0.8	0.021±0.002
12: Tebuconazol-50	8	481±62	3.83±0.22	0.66±0.03	0.55±0.11	1.92±0.41	1.23±0.19	0.16±0.02	12.7±1.9	0.024±0.004
13: Procymidon-12.5	8	481±62	4.02±0.43	0.73±0.09	0.52±0.10	1.95±0.25	1.21±0.21	0.18±0.06	12.3±1.3	0.021±0.003
14: Procymidon-50	6	481±47	4.03±0.35	0.72±0.07	0.43±0.19#	1.73±0.25	1.16±0.22	0.21±0.07	11.5±2.0**.#	0.020±0.004

Rel. organ weights. g per 100 g	N	Testis	Epididymis	Prostate	Seminal vesicle	LABC	Bulbo	Liver	Thyroid
1: Control	16	0.79±0.06	0.14±0.01	0.13±0.03	0.39±0.07	0.28±0.04	0.044±0.014	2.67±0.19	0.0045±0.0006
2: Pestimix-14.58	18	0.85±0.08	0.15±0.02	0.15±0.03	0.43±0.08	0.28±0.04	0.053±0.017	2.55±0.15	0.0053±0.0022
3: Pestimix-29.17	12	0.86±0.14	0.16±0.02	0.13±0.02	0.45±0.10	0.27±0.04	0.038±0.013	2.55±0.13	0.0053±0.0010
4: Pestimix-43.75	16	0.87±0.06	0.15±0.02	0.10±0.04	0.44±0.08	0.25±0.04	0.046±0.012	2.59±0.17	0.0041±0.0012
5: Epoxiconazol-3.75	10	0.86±0.06#	0.14±0.02	0.14±0.03	0.42±0.09	0.27±0.04	0.046±0.010	2.55±0.21	0.0042±0.0006
6: Epoxiconazol-15	2	0.97±0.06#	0.18±0.01#	0.15±0.04	0.50±0.04	0.31±0.004	0.070±0.019#	2.75±0.22	0.0060±0.0007#
7: Mancozeb-6.25	8	0.83±0.09	0.15±0.02	0.11±0.05	0.39±0.09	0.28±0.06	0.048±0.011	2.60±0.09	0.0062±0.0037
8: Mancozeb-25	10	0.82±0.08	0.15±0.01	0.13±0.02	0.40±0.10	0.26±0.02	0.043±0.014	2.69±0.19	0.0060±0.0032
9: Prochloraz-8.75	10	0.85±0.07	0.15±0.02	0.13±0.03	0.42±0.06	0.27±0.04	0.056±0.013	2.61±0.22	0.0044±0.0006
10: Prochloraz-35	6	0.84±0.04	0.15±0.01	0.15±0.03	0.45±0.05	0.29±0.03	0.052±0.025	2.43±0.18#	0.0046±0.0004
11: Tebuconazol-12.5	8	0.86±0.09	0.15±0.03	0.13±0.03	0.41±0.09	0.28±0.04	0.039±0.015	2.52±0.14	0.0046±0.0003
12: Tebuconazol-50	8	0.80±0.07	0.14±0.01	0.12±0.03	0.41±0.11	0.26±0.05	0.033±0.002	2.63±0.18	0.0051±0.0009
13: Procymidon-12.5	8	0.84±0.11	0.15±0.03	0.11±0.03	0.41±0.08	0.26±0.06	0.038±0.015	2.57±0.15	0.0043±0.0007
14: Procymidon-50	6	0.84±0.05	0.15±0.01	0.09±0.04#	0.36±0.05	0.24±0.03	0.042±0.013	2.37±0.25#	0.0042±0.0007

Table 3.19 Extended Mixture Study. Prostate histology adults. No statistically significant differences were observed between between controls and exposed groups

Prostate histology adults	Number of animals evaluated	Inflammation score 1	Inflammation score 2	Inflammation score 3	Inflammation score 4	Intraepithelial vacuolation
		56%	6%	31%	6%	56%
1: Control	16	9/16	1/16	5/16	1/16	9/16
		67%	11%	11%	11/%	50%
2: Pestimix-14,58	18	12/18	2/18	2/18	2/18	9/18
		58%	25%	8%	8%	67%
3: Pestimix-29,17	12	7/12	3/12	1/12	1/12	8/12
		50%	31%	13%	6%	38%
4: Pestimix-43,75	16	8/16	5/16	2/16	1/16	6/16
		80%	20%	0%	0%	80%
5: Epoxiconazol-3,75	10	8/10	2/10	0/10	0/10	8/10
		0%	50%	50%	0%	50%
6: Epoxiconazol-15	2	0/2	1/2	1/2	0/2	1/2
		63%	13%	13%	13%	38%
7: Mancozeb-6,25	8	5/8	1/8	1/8	1/8	3/8
		90%	10%	0%	0%	40%
8: Mancozeb-25	10	9/10	1/10	0/10	0/10	4/10
		50%	20%	20%	10%	40%
9: Prochloraz-8,75	10	5/10	2/10	2/10	1/10	4/10
		83%	0%	0%	17%	50%
10: Prochloraz-35	6	5/6	0/6	0/6	1/6	3/6
		75%	0%	13%	13%	75%
11: Tebuconazol-12,5	8	6/8	0/8	1/8	1/8	6/8
		75%	0%	13%	13%	25%
12: Tebuconazol-50	8	6/8	0/8	1/8	1/8	2/8
		75%	0%	13%	13%	75%
13: Procymidon-12,5	8	6/8	0/8	1/8	1/8	6/8
		60%	20%	0%	20%	50%
14: Procymidon-50	4 to 5[&]	3/5	1/5	0/5	1/5	2/4

[&]One animal excluded from evaluation except inflammation because of severe lesions

Table 3.20 Extended Mixture Study. Prostate histology adults. Statistically significant differences between controls and exposed are marked with astericks. *indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001

Prostate histology adults	Number of animals evaluated	Focal aciniar atrophy	Epithelial atrophy score 0	Epithelial atrophy score 1	Epithelial atrophy score 2	Acini mainly lined by columnar epithelium	Acini mainly lined by cuboidal epithelium	Acini mainly lined by simple squamous to cuboidal epithelium	Epithelial infolding score 1	Epithelial infolding score 2	Epithelial infolding score 3
1: Control	16	13% 2/16	31% 5/16	25% 4/16	44% 7/16	31% 5/16	6% 1/16	63% 10/16	50% 8/16	25% 4/16	25% 4/16
2: Pestimix-14,58	18	22% 4/18	28% 5/18	56% 10/18	17% 3/18	22% 4/18	11% 2/18	67% 12/18	11%* 2/18	72%** 13/18	17% 3/18
3: Pestimix-29,17	12	8% 1/12	25% 3/12	58% 7/12	17% 2/12	25% 3/12	17% 2/12	58% 7/12	17% 2/12	58% 7/12	25% 3/12
4: Pestimix-43,75	16	6% 1/16	81%** 13/16	13% 2/16	6%* 1/16	56% 9/16	19% 3/16	25% 4/16	19% 3/16	44% 7/16	38% 6/16
5: Epoxiconazol-3,75	10	0% 0/10	40% 4/10	20% 2/10	40% 4/10	50% 5/10	0% 0/10	50% 5/10	30% 3/10	30% 3/10	40% 4/10
6: Epoxiconazol-15	2	0% 0/2	50% 1/2	50% 1/2	0% 0/2	100% 2/2	0% 0/2	0% 0/2	0% 0/2	50% 1/2	50% 1/2
7: Mancozeb-6,25	8	38% 3/8	50% 4/8	25% 2/8	25% 2/8	25% 2/8	25% 2/8	50% 4/8	38% 3/8	25% 2/8	38% 3/8
8: Mancozeb-25	10	40% 4/10	30% 3/10	30% 3/10	40% 4/10	10% 1/10	10% 1/10	80% 8/10	40% 4/10	50% 5/10	10% 1/10
9: Prochloraz-8,75	10	0% 0/10	30% 3/10	50% 5/10	20% 2/10	40% 4/10	10% 1/10	50% 5/10	0%** 0/10	100%*** 10/10	0% 0/10
10: Prochloraz-35	6	0% 0/6	17% 1/6	67% 4/6	17% 1/6	17% 1/6	33% 2/6	50% 3/6	17% 1/6	50% 3/6	33% 2/6
11: Tebuconazol-12,5	8	0% 0/8	50% 4/8	38% 3/8	13% 1/8	38% 3/8	38% 3/8	25% 2/8	13% 1/8	38% 3/8	50% 4/8
12: Tebuconazol-50	8	13% 1/8	25% 2/8	50% 4/8	25% 2/8	25% 2/8	25% 2/8	50% 4/8	13% 1/8	63% 5/8	25% 2/8
13: Procymidon-12,5	8	0% 0/8	50% 4/8	50% 4/8	0% 0/8	50% 4/8	13% 1/8	38% 3/8	0% 0/8	50% 4/8	50% 4/8
14: Procymidon-50	4	25% 1/4	50% 2/4	25% 1/4	25% 1/4	50% 2/4	2% 0/4	50% 2/4	50% 2/4	25% 1/4	25% 1/4

Table 3.21 Extended Mixture Study. Prostate histology in adults mated day 1, 2 or 3 before section or not mated. Statistically significant differences between controls and exposed are marked with astericks. *indicates p<0.05; ** indicates p<0.01

Prostate histology adults	Number of animals evaluated	Epithelial atrophy score 0	Epithelial atrophy score 1	Epithelial atrophy score 2	Acini mainly lined by columnar epithelium	Acini mainly lined by cuboidal epithelium	Acini mainly lined by simple squamous to cuboidal epithelium	Epithelial infolding score 1	Epithelial infolding score 2	Epithelial infolding score 3
Not mated	49	24% 12/49	41% 20/49	35% 17/49	22% 11/49	12% 6/49	65% 32/49	32% 16/49	53% 26/49	14% 7/49
Mated	87	48%** 42/87	37% 32/87	15%** 13/87	41%* 36/87	16% 14/87	43%** 37/87	16%* 14/87	48% 42/87	35%** 31/87

Table 3.23 Extended Mixture Study . Liver histology adult males.

Liver histology adult males	Number of animals evaluated	Inflammatory cell infiltrations			Pigment	Minimal hepatocyte vacuolation
		Portal areas	Centrilobular	Parenchymal		
1: Control	16	16/16 100%	0/16 0%	10/16 63%	0/16 0%	11/16 69%
2: Pestimix-14,58	18	18/18 100%	3/18 17%	11/18 61%	1/18 6%	6/18 33%
3: Pestimix-29,17	12	12/12 100%	0/12 0%	4/12 33%	0/12 0%	2/12 17%
4: Pestimix-43,75	16	16/16 100%	1/16 6%	9/16 56%	0/16 0%	4/16 25%
5: Epoxiconazol-3,75	10	10/10 100%	1/10 10%	5/10 50%	0/10 0%	5/10 50%
6: Epoxiconazol-15	2	2/2 100%	0/2 0%	2/2 100%	0/2 0%	1/2 50%
7: Mancozeb-6,25	8	8/8 100%	0/8 0%	3/8 38%	0/8 0%	3/8 38%
8: Mancozeb-25	10	10/10 100%	0/10 0%	4/10 40%	0/10 0%	3/10 30%
9: Prochloraz-8,75	10	10/10 100%	0/10 0%	3/10 30%	1/10 10%	3/10 30%
10: Prochloraz-35	6	6/6 100%	0/6 0%	2/6 33%	0/6 0%	2/6 33%
11: Tebuconazol-12,5	8	8/8 100%	0/8 0%	5/8 63%	0/8 0%	3/8 38%
12: Tebuconazol-50	8	8/8 100%	0/8 0%	3/8 38%	0/8 0%	1/8 13%
13: Procymidon-12,5	8	8/8 100%	0/8 0%	4/8 50%	0/8 0%	3/8 38%
14: Procymidon-50	6	6/6 100%	0/6 0%	2/6 33%	0/6 0%	2/6 33%

Summary

The objective was to explore if combined developmental exposure to endocrine disrupting pesticides lead to adverse developmental toxicity effects in rats. The pesticides were epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone. The mixture caused adverse effects on male sexual development and gestation length in the dams at dose levels below NOAELs for the single pesticides. Investigations of the blood levels in rat offspring indicated that the mixture caused higher blood levels than exposure to the single pesticides. The mixture effects were well predicted by dose-addition. The survey of dietary cumulative intake did not show for concern for Danish consumers, but it is uncertain, whether the uncertainty factor used to allow room for exposure to other endocrine disrupting chemicals was sufficient. The results indicate that the ADIs are not sufficiently low to protect against the mixture effects. Overall, the results imply that risk assessment based on NOAELs for single chemicals can underestimate the risk and that there is a need for modification of risk assessment procedures for pesticides to take account of mixture effects.

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