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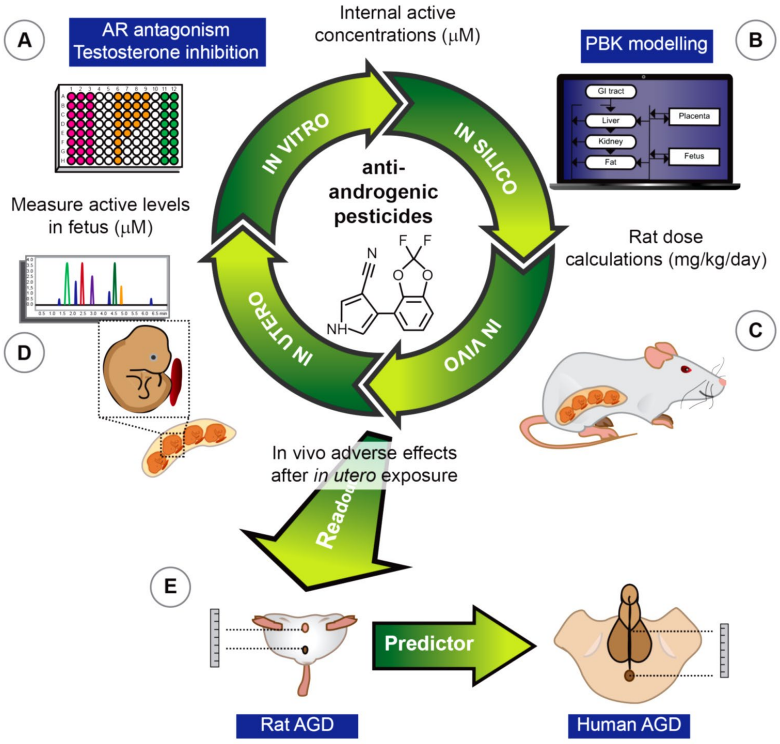
Prediction of persistent health effects caused by widely used anti-androgenic pesticides



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Content

Foreword

The research project ‘Prediction of persistent health effects caused by widely used anti-androgenic pesticides was carried out from 2013 to 2016 and was a collaboration between the research groups on Molecular Toxicology and Reproductive Toxicology from DTU Food and Andreas Kortenkamp’s research group from Department of Life Sciences, Brunel University.

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A steering committee for the project was established and chaired by Jørn Kirkegaard until January 2015 and thereafter by Henrik Frølich Brødsgaard, the Danish Environmental Protection Agency (DEPA)

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 Henrik Leffers, Susanne Hougaard, Rikke Donchil Holmberg, and Martin Larsson for their constructive comments during the writing process of this report.

Summary

Although some pesticides have been shown to have endocrine disrupting effects, the majority of approved active pesticides, some 353 in the EU, have not been tested for sensitive endocrine endpoints such as anogenital distance (AGD) or nipple retention. Since it is not feasible to test all of these pesticides by using in vivo screening approaches within the foreseeable future, the development of alternative test methods for predicting endocrine disrupting effects is imperative. In this context, the morphometric measure of anogenital distance (AGD) has proven to be a non-invasive, life-long biomarker for adverse effects on male reproductive health in animals and humans. AGD can be measured in young offspring and is considered to be an adverse effect, and has thus been included in the OECD guideline for the extended one-generation study in rats.
The major aims for this project, termed 'Prediction of persistent health effects caused by widely used anti-androgenic pesticides' were:

1) To develop a novel approach based on in vitro profiling in combination with physiologically-based kinetic (PBK) modelling, for predicting effects on AGD and other markers of antiandrogenic action
2) To validate the approach in vivo in a rat study
3) To investigate long term endocrine effects in vivo of a selected pesticide

Nine pesticides (dimethomorph, cyprodinil, fludioxonil, fenhexamid, λ-cyhalothrin, azinphos-methyl, quinoxyfen, pyrimethanil and pirimifos-methyl), previously found to antagonize the androgen receptor (AR) in vitro, were profiled for two other mechanisms involved in male reproductive health effect, such as inhibition of testosterone and prostaglandin D synthesis. Relatively simple PBK models, including a fetal compartment, were developed for the pesticides and were used to predict the in vivo doses necessary to obtain the expected critical concentrations in the rat fetus. In vitro activity data, the PBK modelling output, in combination with previous knowledge on no- or low-observed adverse effect levels from 1 and 2 generation rat studies, was compiled into an overview diagramme for the nine pesticides. Based on these diagrams, three pesticides (fludioxonil, cyprodinil, dimethomorph) were selected for the rat developmental study. Fludioxonil was a major AR antagonist (IC_{50}= 2.6 μM) and an inhibitor of testosterone synthesis (IC_{50}= 6.7 μM), cyprodinil inhibited testosterone synthesis (IC_{50}= 6.1 μM) and antagonized the AR (IC_{50}= 28 μM), whereas dimethomorph predominantly antagonized the AR (IC_{50}= 0.9 μM).
In vivo doses were selected based on critical active concentration in the rat fetus whilst ensuring that no maternal or pup toxicity would occur. Fludioxonil and cyprodinil were given at doses of 20, 60, and 180 mg/kg/day and dimethomorph at 6.7, 20 and 60 mg/kg/day to pregnant rats from gestational day (GD) 7 to postnatal day 17. A statistically significant AGD reduction in males was measured at the medium dose of fludioxonil and cyprodinil and at the two lowest doses of dimethomorph, indicating an endocrine disrupting effect of all three pesticides in vivo. AGD was also reduced in females, but the underlying causes remain unclear. No significant effects were found on other morphological markers for endocrine disruption such as nipple retention or weight of reproductive organs.
In a satellite rat GD21 fetal study, pesticides and their metabolites were measured in fetal plasma from both genders, amniotic fluids from pooled litters, as well as in plasma from the dams. Of the three pesticides dimethomorph was metabolized to the least extent. Relatively similar metabolite profiles were found in the dams, fetuses (males and females) and amniotic fluids. The fetal concentrations of the parent compounds were measured in the range 2.3 - 4.3 μM (at 60mg/kg fludioxonil), 5.5 – 7.6 μM (at 60 mg/kg cyprodinil) and 1.3- 1.4 μM (at 20 mg/kg

dimethomorph). These measured concentrations agreed well with those predicted by PBK modeling, with fludioxonil concentrations underestimated by 20%, cyprodinil by 50% and dimethomorph overestimated by around 10%.

In conclusion, we have developed a first proof-of-principle approach combining in vitro and PBK modelling that enables the prediction of effects on male reproductive health. This concept may be used in the future for prioritizing the 353 pesticides (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32011R0540) for further in vivo testing.

Fludioxonil was also investigated for potential long-term effects, focusing on male offspring up until around 1 year of age. Circulating hormone levels, testes and prostate gene expression, including some smaller epigenetic profiling, as well as histological assessments of prostates were performed. An observable trend, albeit not statistically significant, towards reduced testosterone levels in the 3 months old male offspring was noted, accompanied by downregulated *Star* mRNA expression in the testes; an upstream protein serving to transport cholesterol into the mitochondria for testosterone synthesis. In the prostates, significant gene expression changes were found in the 3 months old males, but not in the younger pups at PND17. Genes that were dysregulated included key regulatory factors such as *Ar*, *Apc, Cav1, Gstp1* and *Egfr*, all of which have been shown to be dysregulated in transformed prostate tissue.
A restricted analysis to elucidate whether an epigenetic mechanism was involved in the prostate effects was performed on the promoters *Apc*, *Ar*, *Cav1* and *Gstp1*. A screening using methylation-specific PCR protocols targeting known CpG sites was performed. The only significant finding was that the *Gstp1* promoter was more frequently methylated in the exposed than in control animals, suggesting that fludioxonil exposure can cause abnormal methylation of this gene promoter.
However, no significant effects on prostate histology were observed.

In conclusion, we have identified a pesticide, fludioxonil, with effects on male reproductive health; AGD was slightly reduced, and molecular changes in the testis and prostate were found in the older male pups. Furthermore, the previously reported effects on male reproductive health from long term in vivo studies for dimethomorph were confirmed in this study by the observed AGD effect.

Conclusion

The major aims of this project 'Prediction of persistent health effects caused by widely used anti-androgenic pesticides' were:

1) To develop a novel approach based on in vitro profiling combined with physiologically-based kinetic (PBK) modelling to predict effects on anogenital distance and other markers of anti-androgenic activity
2) To validate the approach in vivo in a rat study
3) To investigate long term endocrine effects in vivo of one selected pesticide

The main findings were:

- Eight out of nine pesticides known to antagonize the AR did also affect hormone synthesis in vitro, including testosterone inhibition for seven of the pesticides.
- The PBK modelling approach was successful in predicting internal levels of pesticides in rat fetuses (within a factor of 0.5-1.1)
- The integrated in vitro/PBK modelling approach, together with information from risk assessment reports, was valuable for ranking and selecting pesticides for in vivo testing
- Analytical chemistry was successful in measuring parent pesticides and their metabolites in dams, fetuses and amniotic fluid. Metabolite profiles were judged as relatively similar between dams, female and male pups.
- Three pesticides, fludioxonil, cyprodinil and dimethormorph, were predicted to affect AGD in vivo at non-toxic doses, and all caused a significant reduction of AGD in male rats (although without a clear monotonic dose-response relationship). The combination of a plausible mode of action underlying the AGD changes and previously reported changes on male reproductive organ weights (DAR) suggest that the observed AGD effects are non-random findings.
- No other morphological or histological markers of anti-androgenic actions were judged as statistically significant.
- At the molecular level, fludioxonil affected gene expression in testis (Star) and prostates (e.g. Ar) and caused a 50% reduction in testosterone (albeit not statistically significant).
- Histological analysis provided no indications for prostate cancer in adult rats after prenatal exposure to fludioxonil, but signs of endocrine disruption in prostates of young rats call for further investigation of potential persistent adverse effects.
- Limited analyses of epigenetic changes of affected genes in the prostates failed to identify a causative relationship.However, since the methylation analysis was targeted rather than global, these data does not exclude the involvement of epigenetic changes as causative mechanisms.

In conclusion, we have developed a first proof-of-principle showing that it is possible to predict effects on AGD by a combined in vitro and PBK modelling approach. Such a concept may be used in the future for prioritizing the untested 353 pesticides (and other chemicals) for in vivo testing.

Furthermore, three pesticides, fludioxonil, dimethomorph and cyprodinil, were identified with multiple targets in vitro (such as AR antagonism, affected sex steroid hormone production) and a weak endocrine disrupting effect in vivo.

1. Context and background

Male reproductive health is defined by both proper development of the reproductive system and by maintenance of reproductive function throughout adult life, including the capacity to reproduce. Male sexual development starts with the differentiation of the bipotential gonads into testes, a process that occurs independently of sex hormones (Svingen & Koopman, 2013). Subsequently, the testes produce androgens which drive secondary sex differentiation, ensuring proper masculinization of the male fetus (Sharpe, 2006). Consequently, the male fetus is vulnerable to endocrine-disrupting chemicals (EDCs) since they can interfere with androgen synthesis or function and thereby disrupt normal development.
Many pesticides have been shown to possess endocrine disrupting properties. According to WHO the definition of 'An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations'.
An increasing body of evidence suggests that exposure to such pesticides during prenatal and early life can cause reproductive disorders in humans, which can manifest for instance as cryptorchidism, hypospadias or decreased penile length in new-born boys after intrauterine exposure (Damgaard et al., 2006; Andersen et al., 2008; Rocheleau et al., 2009). This is an important issue since male fertility has reportedly been declining for many years in many countries (Andersson et al., 2008; Jorgensen et al., 2012), but also since perinatal hypospadias/cryptorchidism are risk factors for reduced sperm quality and testicular cancer in adulthood (Skakkebæk et al., 2001).

Several studies on the abandoned organochlorine pesticides and current-use pesticides have shown associations with male reproductive health effects. For instance, persistent organochlorines such as p,p-DDT, p,p-DDE, B-HCH, HCB, α-endosulfan, cis-HE, oxychlordane, dieldrin, which are now banned in most countries, have been detected in all tested breast milk samples of mothers in Denmark and Finland. Notably, levels were significantly higher in mothers that had given birth to sons with cryptorchidism than in matched controls (1997-2001, Damgaard et al. 2006). Also, female Danish greenhouse workers exposed to currently used pesticides were more likely to give birth to a son with cryptorchidism than random mothers from the Copenhagen area (6.2% versus 1.9%). Furthermore, sons of mothers who directly handled treated plants or were otherwise engaged in spraying pesticides, had significantly smaller penises than sons born from mothers not working in the greenhouse industry (Andersen et al. 2008). Finally, a meta-analysis including studies from USA and Europe reported that maternal occupational exposure to pesticides was associated with a 36% increased risk of hypospadias relative to the risk in mothers without exposure (risk ratio: 1.36; confidence interval: 1.04-1.77) (Rocheleau et al. (2009). However, another meta-analysis combined the results from all relevant epidemiological studies published since 2006 and found no evidence for a robust, clinically significant association between any pesticide exposure and cryptorchidism or hypospadias (Ntzanti et al.2013).

Fifteen years ago the Testicular Dysgenesis Syndrome (TDS) hypothesis was proposed, linking a rise in common male reproductive disorders such as cryptorchidism, hypospadias, reduced sperm quality and testicular cancer with an increasing exposure to EDCs (Skakkebæk et al., 2001). Since then there has been a significant number of studies showing associations between TDS and exposure to various chemicals, including pesticides. In vitro hormone receptor screening suggests a preponderance of anti-androgenic activity compared to estrogenic activity in non-organochlorine (current-use) pesticides (Kojima et al. 2004; Orton et al., 2009). For several pesticides there is a good correlation between androgen receptor (AR) antagonist properties in vitro and in vivo anti-androgenic effects in male rats after developmental exposure. Anti-andro-

genic effects both in vitro and in vivo following maternal exposure have been reported for several pesticides including the herbicide linuron (Wolf et al, 1999; Lambright et al., 2000), the fungicides prochloraz (Vinggaard et al., 2005), procymidone (Ostby et al., 1999; Hass et al., 2007), tebuconazole (Taxvig et al., 2007), vinclozolin (Uzumcu et al., 2004; Anway et al., 2006; Hass et al., 2007), the organochlorine insecticides DDE (Wolf et al., 1999) and endosulfan (Sinha et al., 2001), the organophosphate dimethoate (Verma and Mohanty, 2009), and the pyrethroid insecticide deltamethrin (Andrade et al., 2002).

Shortening of the anogenital distance (AGD) in male offspring has proved to be a valuable biomarker for TDS development in humans and animals (Thankamony et al, 2014; Dean and Sharpe 2013) as a reduced AGD indicates impaired androgen levels or function during early development. A short AGD is associated with malformation of sex organs (cryptorchidism, hypospadias, penile length) and poor sperm quality in animals, as well as in humans. Thus AGD - together with NR - are now included in an OECD test guideline as an adverse effects linked to male reproductive health. A shorter AGD has also been associated with increased prostate cancer risk in humans (Castaño-Vinyals et al., 2012) and in animals, where perinatal exposure to a mixtures of anti-androgenic pesticides caused an increased frequency of age-related changes in the prostates (Isling et al., 2014).
Reduced androgen levels or function is believed to be the main mode of action leading to reduced AGD and NR in male offspring. EDCs can interfere with androgen-dependent mechanisms and affect the health of the male reproductive system in multiple ways including direct AR antagonism, changes in androgen synthesis, metabolism and clearance, feedback regulation, and AR expression in target organs. Many pesticides have been found to block the AR (Kelce et al., 1995; Ostby et al., 1999; McKinnell et al., 2001; Bonefeld-Jørgensen et al., 2007). However, there are strong indications that we may only have seen the tip of the iceberg in terms of the number of chemicals capable of producing these effects. By applying a quantitative structure–activity relationship (QSAR) model for AR antagonism, we predicted that out of 82,800 existing chemicals, 8% were positives as AR antagonists (Vinggaard et al., 2008), indicating that many chemicals may be able to antagonize AR, if they reach critical tissue targets during periods of heightened vulnerability in fetal life.

Recently, it was suggested that also prostaglandin synthesis inhibition might be a target for EDCs, as the drug paracetamol was shown to cause anti-androgenic effects in epidemiological studies as well as in a rat developmental study (Kristensen et al., 2011) and as many pesticides were found being able to inhibit prostaglandin D2 synthesis (Kugathas et al. 2016).

Yet another mechanism suspected of being involved in chemically-induced male reproductive health problems is through epigenetic marks that determine the functional output of the information stored in the genome. Delayed adverse health effects like for instance persistent effects on reproductive development in sons born by women working within gardening have been reported and these effects are conceivably brought about by leaving epigenetic marks (Andersen et al., 2008; Skinner et al., 2010; Collotta et al., 2013). More knowledge is needed on whether pesticides have the ability to permanently change the way genes are expressed in the mammalian organism by epigenetic mechanisms and in-depth knowledge on the exact molecular mechanism at the promoter site is needed as well. Epigenetic effects are especially important as these in some cases may lead to transgenerational effects.

Endocrine relevant data on currently used pesticides is minimal, and most of the 353 current-use pesticides in the EU have not been tested thoroughly for endocrine disrupting effects. Testing in animal studies will not be attained in foreseeable future and alternative methods to prioritize pesticides for in vivo testing are greatly needed. The aim of this project was first of all to develop a proof-of-principle based on in vitro testing and physiologically-based kinetic (PBK) modelling to predict a reduced AGD and other markers of anti-androgenic action of pesticides and

secondly to investigate long term effects caused by an exposure to one selected pesticide during fetal life including development of prostates and testes.

2. Research Plan

The basis for this project was the identification of a number of currently used pesticides as in vitro AR antagonists (Orton et al. 2011). Human exposure data formed the basis for selection of the pesticides to be tested for anti-androgenic activity in vitro, focusing on pesticides to which humans are most likely exposed. European databases were used to select 134 candidate pesticides, followed by a filtering step according to known or predicted receptor-mediated anti-androgenic potency, based on our in-house developed QSAR model. In total, 37 pesticides were tested for in vitro AR antagonism. Here, 24 of the 37 pesticides were identified as anti-androgenic, and among them 9 pesticides had not previously been reported as being anti-androgenic (dimethomorph, cyprodinil, fludioxonil, fenhexamid, λ-cyhalothrin, azinphos-methyl, quinoxyfen, pyrimethanil and pirimifos-methyl). Due to their demonstrated in vitro anti-androgenic potency, current use and estimated exposure of humans, but lack of adequate in vivo data from animal experiments, we came to the conclusion that there is an urgent need for further evaluation of these pesticides for anti-androgenic effects. In addition to the newly identified anti-androgenic pesticides, we included two additional pesticides in the current study, imazalil and o-phenylphenol, both known as potent in vitro AR antagonists, but so far not tested for sensitive anti-androgenic effects such as AGD in vivo.

We aimed to develop an alternative approach for evaluating effects on male reproductive health of these pesticides. The approach was based on the hypothesis that an integration of in vitro data with physiologically-based kinetic (PBK) modelling can be used for prioritizing pesticides for thorough in vivo developmental studies. Our plan was to supplement the previously accrued AR antagonism data with investigations of the selected pesticides on two other relevant anti-androgenic mechanisms, i.e. inhibition of androgen synthesis in vitro and inhibition of prostaglandin D2 synthesis. Data from these endpoints were considered as minimum information necessary to evaluate a potential in vivo potency of the pesticides, especially with respect to avoiding false-negative judgments. Here, modelled fetal concentrations were compared with in vitro chemical potency to pinpoint anti-androgens unlikely to produce in vivo effects since critical tissue concentrations cannot be reached. Three pesticides were selected for further in vivo testing, with the experimental focus on detecting changes in the AGD or other endocrine-related responses in males. Based on potential significant effects observed for AGD in newborn males, one pesticide was selected for a long-term effect study lasting until 1 year of age. The remaining male pups and all female pups were killed at PND17. Persistent health effects in the adult males were looked for including prostate histology as well as epigenetic changes manifested as changes in DNA methylation in prostates and testes (see fig. 1 for an overview of the work flow of the project).

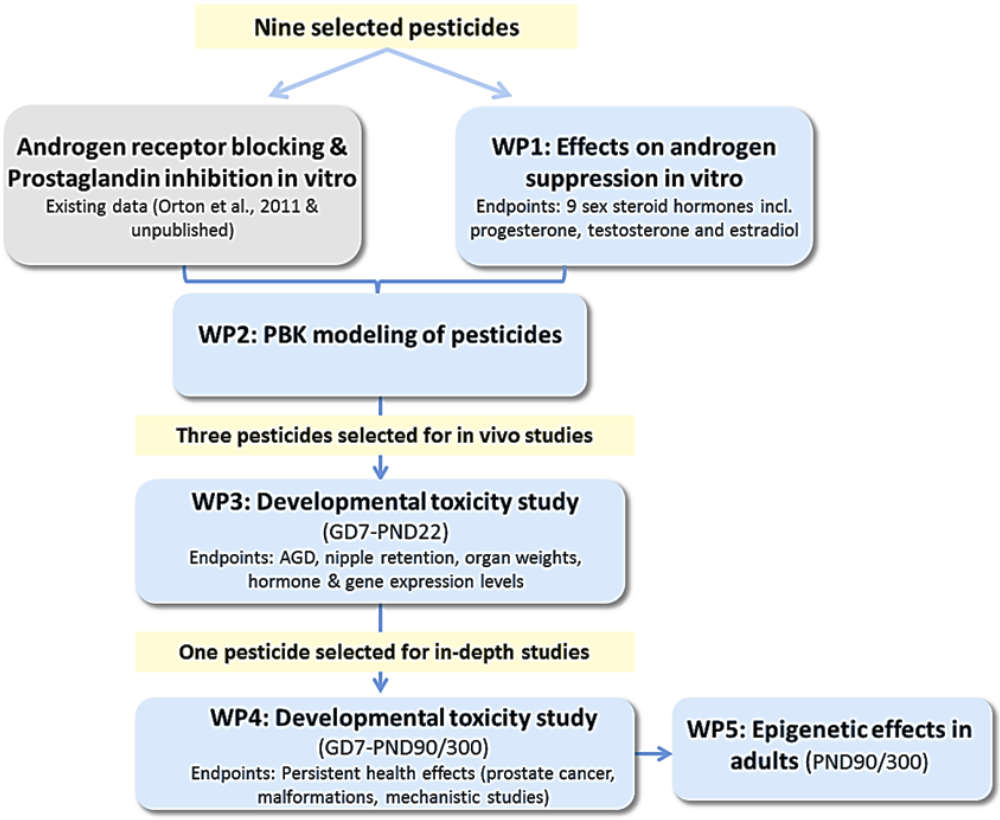

FIGURE.1: The work flow of the project

3. Hypotheses

The overall aims of the project were: 1) To develop a scheme whereby animal use in reproductive and developmental toxicity testing of pesticides can be reduced by prioritizing those substances for in vivo testing that are most likely to induce adverse endocrine disrupting effects in whole animals. This requires knowledge about the applicability of alternative test methods such as in vitro studies and PBK modelling as new testing strategies. 2) To investigate long-term health effects of one selected pesticide.
The realization of these goals required the examination of the following specific hypotheses:

1. It is possible to develop generic PBK models for pesticides with an anti-androgenic mode of action that are capable of simulating measured fetal concentrations in rats after repeated oral gavage?
2. In vivo doses of anti-androgenic agents that will result in fetal concentrations associated with in vitro anti-androgenic activity can be predicted (reverse dosimetry).
3. Pesticides with high in vitro anti-androgenic activity and expected to reach the fetus at non-toxic intake doses will produce anti-androgenic effects in rodents (In vivo extrapolation).
4. Persistent epigenetic effects in terms of DNA methylation will be induced in adult rat offspring after perinatal exposure to a male developmental toxicant (epigenetics).
5. Perinatal programming by exposure to an anti-androgenic pesticide can induce persistent changes in the prostate (thus predisposing the gland to elevated cancer risk).

4. Theoretical basis & methods

4.1 Steroidogenesis assay

The H295R steroidogenesis assay has been adopted as an OECD test guideline (TG441). The assay is an in vitro model for the investigation of effects on steroid hormone synthesis, and is based on the H295R human adrenocortical carcinoma cell line, which expresses all the key enzymes necessary for steroid hormone production. The H295R cells represent a unique in vitro system in that they have the ability to produce all the steroid hormones found in the adult adrenal cortex and the gonads allowing testing for effects on both corticosteroid synthesis and the production of sex steroid hormones such as androgens and estrogens (Hecker et al., 2011). The protocol used was as described by Rosenmai et al. 2014.

4.2 PBK modelling

The objective was to develop a PBK modelling approach with respect to the 'chemical space' of anti-androgens and specific developmental endpoints by evaluating existing PBK models. Anti-androgens comprise a diverse group of chemicals with widely variable physico-chemical properties. It is thus a challenge to devise a modelling strategy that can incorporate all these features. The second specific feature of the model to be developed was its emphasis on the fetal compartment and the timing of developmental steps important in male sexual differentiation.
A 'forward' approach was used for developing and evaluating the PBK models, using the in vivo dose-response data for anti-androgenic effects in the rat for known chemicals as input values. Based on these data, the levels in the fetal compartment were modeled and compared to the concentrations associated with in vitro effects.
The following chemicals, representing a diverse range of chemical structures and physico-chemical properties, were selected for investigation: flutamide, procymidone, vinclozolin, prochloraz, DEHP, DDE and bisphenol A.
Pesticides with in vitro anti-androgenicity were evaluated for their potential to induce in vivo effects. A 'reverse' approach was adopted when in vitro data was the model input. It was then evaluated whether concentrations in the fetal compartment causing in vitro effects require doses to the dams so high that thresholds for maternal toxicity, or the solubility limits for the tested chemicals, were exceeded. If so, in vivo effects were deemed unlikely. In all other cases, in vivo effects were judged likely and the chemical deemed a candidate for in vivo testing.
Physiological and kinetic parameters such as absorption, distribution and clearance parameters related to metabolism and excretion pathways, were retrieved from peer-reviewed compilations of ranges and reference values for laboratory animals. For rates relevant to the fetal compartment, published data-based models were consulted. Tissue/blood partition coefficients were estimated by using in silico models. For compound specific parameter values, relevant data compilations were consulted. More details on PBK modelling can be found in *Appendix 2*.

4.3 In vivo developmental toxicity study

The rat reproductive/developmental toxicity screening test is considered sensitive for identification of in vivo active anti-androgenic compounds. The OECD guideline (TG 421) includes premating exposure to chemicals, but in this project a modified version was applied. This model is based on exposure of rats during gestation and lactation and investigations of a range of parameters in the newborn and pre-pubertal offspring. In this project, a subgroup of pups was kept to adulthood for examination of long-term persistent effects.

In brief, groups of time-mated Wistar rats (n=10-14) were exposed during gestation and lactation to the selected compounds at 3 dose levels. Doses were selected on the basis of historical 1 or 2 generation studies and on the outcome of PBK modelling in the “reverse” mode. Male offspring was examined for effects on selected markers of anti-androgenic activity.
In addition, epigenetic effects were assessed in this model by investigating adult offspring for persistent gene expression changes. This was performed in two target tissues, the prostate and the testes. Also gene expression analysis was conducted to determine mechanisms of action of the selected pesticides in terms of interference with androgen action

4.3.1 Design of animal study

110 time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day 3 (GD 3) of pregnancy. The study was performed using 3 blocks of 34–42 dams (separated by 1 week), and all dose groups were equally represented in the blocks. The study was performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee. The animals were housed in pairs until GD 17 and alone thereafter under standard conditions as described in (Christiansen et al 2014).
On the day after arrival (GD 4), the time-mated dams were distributed into ten groups with similar weight distributions in all groups. The group size was 14 in the control group, 12 in the middle dose groups and 10 in low and high dose groups. However, not all dams appeared to be pregnant. Dams were dosed by gavage during morning hours from GD 7 to GD 21 and from the day after birth to pup day (PD) 16. The dams received vehicle (corn oil) or one of the nine pesticide doses: dimethomorph 6.7, 20 or 60 mg/kg/day, cyprodinil 20, 60 or 180 mg/kg/day, or fludioxonil of 20, 60 or 180 mg/kg/day (Flu20, Flu60 or Flu180).
At GD 21 seven dams were sacrificed; one from the control group and two from each group exposed to the middle dose of fludioxonil, cyprodinil or dimethomorph. Uteri were taken out, and the number of live fetuses, location in uterus, resorptions, and implantations were registered. Body weight, sex and any anomalies were recorded. Amniotic fluid, the blood of the dams and pooled trunk blood from all female and male fetuses in each litter were collected, and stored at −80 °C until it was used for analysis of pesticides and their metabolites, by means of LC-QTOF.
Pup weight and AGD was measured at birth in all male and female pups. AGD was measured as the distance between the genital papilla and the anus using an ocular stereomicroscope. Nipple retention was measured in all pups at PD 14 (Christiansen et al., 2014). The measurements were performed blinded with respect to treatment group by a skilled technician. At PD 16, one male pup per litter were sacrificed and body weights and weights of testes, epididymides, ventral prostate, seminal vesicle, levator ani bulbocavernosus muscle and bulbourethral gland were determined.
A subset of male offspring exposed to fludioxonil (and corresponding controls) were weaned at PND 22 and housed under standard conditions, two per cage until either PND 98 (designated 3 months of age) or PND 330 (designated 11 months of age). At these time-points10-20 males per group were sacrificed and examined for changes in prostate weight, histology and gene expression.
Remaining dams and pups exposed to dimethomorph or cyprodinil were sacrificed between PND 17 and 22.

4.3.2 Gene expression analyses

Gene expression profiling was performed on ventral prostate and testicular tissue from male offspring following exposure to the three doses of fludioxonil and compared to the control group.

The fludioxonil study was selected based on the pipeline strategy outlined previously to compliment the morphological and histopathological data in an effort to gain new insights into the molecular and cellular principles underpinning the persistent (or delayed) phenotypic manifestations.

Total RNA was extracted from tissues conserved in RNA-Later and then used to synthesize cDNA following protocols as described previously (Svingen et al 2015). For prostates, an initial experiment using a targeted 84-gene array (RT2 ProfilerTM PCR Array PARN-135ZA; Qiagen) was used to screen for changes in transcript levels of key targets known to be dysregulated in various disease states. Two ventral prostate samples were randomly selected from each of the three exposure groups (Flu20, Flu60, Flu180) plus control at both UD17 and UD98. The array was run using a 384-well format and a 7900HT Fast Real-Time PCR System (Applied Biosystems), with relative transcript levels determined by the 2dCt-method using the Qiagen on-line Data Analysis Center.

Gene specific TaqMan assays were performed for a selection of genes on both prostate and testis tissues from fludioxonil-exposed male offspring and corresponding control groups. For prostate tissues, a subset of 12 genes and two housekeeping genes were selected based on results from the 84-gene array, whereas 10 genes were selected for testis analyses based on knowledge on cell-specific markers and androgen-sensitive targets. Expression profiling was performed on prostate and testis tissues from PND98 and PND330 male rats. All TaqMan assays are listed in Table 1. Assays were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and relative transcript abundance calculated by the 2dCt-method using the reference genes Hprt and Rpl13a.

TABLE 1: TaqMan assays used for gene expression analyses on prostate and testis tissues from male offspring of rats exposed to fludioxonil.

Gene	Name	TaqMan assay
Prostate		
Apc	Adenomatosis polyposis coli	Rn00560714
Ar	Androgen receptor	Rn00560747
Cav1	Caveolin 1	Rn00755834
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	Rn 00589996
Dnmt1	DNA methyltransferase (cytosine-5) 1	Rn00709664
Egfr	Epidermal growth factor receptor	Rn01434447
Egr3	Early growth response 3	Rn00567228
Gstp1	Glutathione S-transferase, pi 1	Rn00561378
Hprt	Hypoxanthine guanine phosphoribosyl transferase	Rn01527840
Odc1	Ornithine decarboxylase, structural 1	Rn01469808
Rpl13a	Ribosomal protein L13A	Rn00821946
Sfrp1	Secreted frizzled-related protein 1	Rn01478472
Socs3	Suppressor of cytokine signaling 3	Rn00585674
Tp53	Tumor protein p53	Rn00755717
Testis		
Ar	Androgen receptor	Rn00560747
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	Laier et al. 2006

Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	Laier et al. 2006
Ddx4	DEAD-box helicase 4	Rn01489814
Hsd3b1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	Rn01774741
Insl3	Insulin-like 3	Rn00586632
Lhcgr	Lutenizing hormone /choriogonadotropin receptor	Rn00564309
Nr5a1	Nuclear receptor subfamily 5, group A, member 1	Rn00584298
Rpl13a	Ribosomal protein L13A	Rn00821946
Sox9	SRY (sex determining region Y)-box 9	Rn01751069
Star	Steroidogenic acute regulatory protein	Laier et al. 2006

4.3.3 Epigenetic investigations

Many studies in humans and animals have shown that altered gene expression in prostate tissues, particularly during oncogenic transformations, can be due to altered methylation status of specific gene promoters (reviewed by Massie et al 2016). Based on our gene expression profiling of prostates from adult rats following in utero exposure to fludioxonil, we performed methylation-specific PCR analyses on four genes that were dysregulated in our study and are further known to have differentially sensitive promoters: Apc, Ar, Cav1 and Gstp1.

The online MethPrimer software (Li & Dahiya, 2002) was used to design primers within a 1500 nt promoter region (-1200 to +300 nt relative to transcription start site) obtained from the UCSC Genome browser. All primers spanned suitable CpG sites and are listed in Table 2. For methylation-specific PCR (MS-PCR), genomic DNA was isolated from ~100 mg prostate tissue and a total of 1 µg gDNA was bisulfide-treated using an EpiTech Bisulfite kit (Qiagen) as per the manufacturer's instructions. MS-PCR was run on a MJ Research PTC-200 Thermal Cycler in 50 µl reaction containing: 1.25U Taq polymerase (Fisher Scientific, Roskilde, Denmark) with accompanying buffer, 200 µM dNTPs, 300 nM each forward and reverse primer, and 1 µL bisulfite-treated DNA. Cycling conditions were: 95 °C for 2 min; followed by 45 cycles of 94 °C for 30 s, 57-59 °C (depending on primer set) for 1 min, 72 °C for 1 min; then and a final extension at 72 °C for 7 min. Amplification products were separated on a 4% NuStore GTG agarose gel containing ethidium bromide and visualized under UV light using a ChemiDoc XRS+ system.

TABLE 2: Gene promoter specific primers used for MS-PCR analyses on prostate tissues from adult male rats after in utero exposure to fludioxonil. M = methylation specific; U = unmethylation specific primers; Tm = melting temperature.

Gene	Primers	Tm
Apc	M-Apc.F 5'TTGAAGATGGAGAATTTAAATTTTC	57.2
	M-Apc.R 5'TAAATAAACTACAATCAAAATCGCA	57.2
	U-Apc.F 5' TTGAAGATGGAGAATTTAAATTTTTG	59.1
	U-Apc.R 5' AATAAATAAACTACAATCAAAATCACA	54.9
Ar	M-Ar.F 5'GTATTTAAGAATAATTGGTAGTCGG	54.8
	M-Ar.R 5'AACAAAAACTTTAACTTAAAACGAT	54.4
	U-Ar.F 5'GGTATTTAAGAATAATTGGTAGTTGG	56.0
	U-Ar.R 5'AAACAAAAACTTTAACTTAAAACAAT	53.8
Cav1	M-Cav1.F 5'TTGAGATGATGTATTGGGAAAATAC	58.4
	M-Cav1.R 5'AAAATTCTAACAACGAAAAACGAA	58.4
	U-Cav1.F 5'TTGAGATGATGTATTGGGAAAATAT	58.0

	U-Cav1.R 5'CAAAATTCTAACAACAAAAAACAAA	56.8
Gstp1	M-Gstp1.F 5'TTTAGTTTTTGGTGTAAGTTGTTCG	58.8
	M-Gstp1.R 5'ACTTTAAATCCACACCTCTATCTACG	58.4
	U-Gstp1.F 5'TTAGTTTTTGGTGTAAGTTGTTTGG	58.7
	U-Gstp1.R 5'AACTTTAAATCCACACCTCTATCTACAC	58.7

4.3.4 Histological analyses

Ventral prostates of adult males were evaluated with regard to degree of inflammation, epithelial atrophy, and atypical hyperplasia. One section was evaluated per animal. Additionally, morphometric examination was performed in ventral prostates from adult males to identify possible changes in the relative areas of different compartments. This was done for controls and Flu60 males at 3 months of age, and for all dose groups at 11 months of age. Each area was applied a grid with 15 points that were manually assigned as epithelium, stroma, lumen or outside tissue section/undefined as described by Boberg et al. 2016.

In the dorsolateral prostate, the anterior, dorsal and lateral parts were evaluated separately with focus on inflammation, epithelial height and morphology, degree of folding of epithelium (lateral prostate mainly) and degree of cell sloughing and cellular atypia (dorsal prostate mainly).

4.3.5 Statistical analysis

GraphPad Prism 5 (Graph Pad Software, La Jolla) was used for analysis of gene expression data and morphometric data, whereas SAS Enterprise Guide 4.3 was used for all other data.

Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Results from each of the three pesticides were analyzed separately, i.e. a comparison of the three doses of each pesticide to control values was performed. AGD and organ weights were analyzed using body weight as covariate. 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. The number of nipple/areolas was assumed to follow a binomial distribution with a response range between 0 and θmax, with θmax being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θmax was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on nipple retention, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method using the SAS procedure PROC GENMOD. Histological scoring data were evaluated using the 2x2 Fisher's Exact Test.

5. Results

5.1 In vitro profiling of the pesticides

Before starting the experimental work we gathered all publicly available information about the selected nine pesticides: data for AR antagonism (Orton et al. 2011) and PGD2 synthesis inhibition (Kugathas et al. 2016) are shown in Table 3, together with NOAEL/LOALs reported for maternal toxicity and/or developmental toxicity from 1 or 2 generation in vivo studies. In general, DARs for fludioxonil, cyprodinil and dimethomorph concluded that these pesticides are not reproductive toxicants. However, prostate and testis changes in dogs as well as Leydig cell tumors have been reported in 2-year studies with dimethomorph and a higher testis weight was seen in some studies with fludioxonil. A score for human exposure has previously been estimated (Orton et al. 2011).

Table 3: Overview of existing data

	AR antagonism (IC_{20} µM) (Orton et al. 2011)	**PGD2 inhibition (IC_{50} µM)** [#]	***In Vivo* NOAEL/LOAL mg/kg bw**[a]	**Human exposure score** (Orton et al. 2011)
Fludioxonil	0.8	30	21 / 212	25
Cyprodinil	15.1	0.8	81 / 326	33
Dimethomorph	0.3	Inactive	21 / 67	12
Imazalil	3.2	1.5	20 / 80	32
Quinoxyfen	4.8	Not tested	20 /110	12
Fenhexamid	2.0	7.4	38 / 406	24
O-Phenylphenol	3.4	0.2	40 / 140	21
λ-Cyhalothrin	23.1	Not tested	2 / 6.7[b]	15
Pyrimethanil	27.2	8.3	23/ 293	28

[a]: The parental values from 2-generation reproductive toxicity studies in rats from EFSA's Draft Assessment Reports (DARs) or public available reports. [b]: The listed values are for Cyhalothrin and not λ-Cyhalothrin.
The highlighted compounds are the ones chosen for *in vivo* testing
[#]Kugathas et al. 2016

5.1.1 Effects on steroidogenesis

Steroid hormone production in the human adrenocortical carcinoma cells was affected by all nine pesticides, but with different profiles (Table 4).

Six of the nine pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyphen, fenhexamide and o-phenylphenol) showed a reduction in testosterone levels, with imazalil and fludioxonil showing a significant reduction already at the lowest tested concentration of 0.8 µM (Table 4, LOECs are shown in parenthesis). Only dimethomorph had a slight effect on androgen levels. Most pesticides also affected levels of other androgens except fludioxonil and o-phenylphenol.

Imazalil and fenhexamid were the only two pesticides having effect on all measured hormone levels (Table 4). For pyrimethanil, no significant effects were found on any of the measured hormones (Table 4) whereas λ-cyhalothrin was only shown to increase glucocorticoids.

The three pesticides subsequently selected for in-depth in vivo studies were ranked according to effects on specific endpoints:

Reduction of androgen levels: fludioxonil > cyprodinil >> dimethomorph

Increase in estrogen levels: dimethomorph> fludioxonil> cyprodinil

Increase in corticosteroid levels: dimethomorph>> fludioxonil > cyprodinil

The concentration response curves of the pesticides on androgens levels (testosterone and androstendione) and estradiol can be found in *Appendix 4*.

Table 4: Overview of the effects of the nine pesticides on sex hormone production in the H295R cell assay

	Testosterone	Androstendione	DHEA	Progesterone	Estradiol	Corticosterone
Fludioxonil	↓(0.8)	-	-	↓(3.1)	↑(3.1)	↑(12.5)[b]
Cyprodinil	↓(6.3)	↓(12.5)	-	↓(1.6)	↑(6.3)	↑ (25)
Dimethomorph	↓(25)	↓(25)	↓(25)	-	↑(0.8)	↑ (0.8)
Imazalil	↓(0.8)	↓(0.8)	↓(0.8)	↑(0.8)	↓(0.8)	↓(0.8)
Quinoxyfen	↓(1.6)	↓(1.6)	↑(1.6)	↑(50)[b]	↑(25)[b]	↑(3.1)
Fenhexamid	↓(6.3)	↓(12.5)	↑(12.5)	↓(12.5)	↑(12.5)	↓ (1.6)
O-Phenylphenol	↓(25)	-	-	-	↑(25)	↑(6.3)
λ-Cyhalothrin	-	-	-	-	-	↑(3.1)[a]
Pyrimethanil	-	-	-	-	-	-

- : no effect; ↑ : increase; ↓ : decrease; **red** indicates decrease in hormone levels; **blue** indicate increased in hormone levels
( ) : LOEC (lowest observed effect concentration)
DHEA: Dehydroepiandrosterone
[a]: increase starting at 3.1µM, decrease from 25 µM
[b]: an increase was seen, but cytotoxicity was also observed at the same concentrations

5.2 Development of the PBK models

5.2.1 Background

PBK models are defined as mathematical models to predict pharmacokinetics, as opposed to empirical approach models. In the latter case, all information regarding the model is derived from the available concentration versus time data and the choice of the pharmacokinetic model is based on statistical criteria and methods.

With PBK modelling, the output is derived from parameters that serve as model input; no fitting of data is involved. However, many of the parameters on which PBK models rely, are not available, and have to be derived by estimation, either from well-established in situ and in vitro predictive tools, or directly from experimental data. Generally, PBK model development is less problematic if it comes to the estimation of species-specific physiological parameters, especially in case of rodents where the literature is likely to provide all essential data, but populating compound-specific kinetic parameters with “good” values is far more demanding and difficult.

For the pesticides we estimated most ADME model parameters from available data summaries provided by the EFSA’s Draft Assessment Reports (DARs), mainly as the pesticide registration requires information about toxicokinetics in mammals, which is typically evaluated in vivo using the rat as test system (OECD TG 417, 2008), including metabolite profiling and identification. Although this seems to be an optimal data situation for any compound-specific in vivo modelling on rodents, their usage for kinetic modelling was often rather limited. For many of the pesticides investigated in this project, data about the tissue distribution for different time points within the first 24 hours was often missing, especially for their metabolites. Generally, data from in vivo studies conducted according to revised versions of TG417 were much more detailed and useful for the modelling.

Unfortunately it proved impossible to quantify the impact of uncertainties from the data sources for the simulated fetal concentrations due to lack of data. Sensitivity analyses, which show how a model response changes under the influence of individual parameter changes, were performed whenever justified. Not only did we analyze each parameter in the PBK model for sensitivity, but also for each day between GD6 and GD19.

This sensitivity analysis recorded the influence of individual parameter-driven variations of the area under the curve (AUC) in the fetal compartment after a single oral dose. The most sensitive parameter for the maternal blood circulation was the GI tract absorption constant, and under the assumption of a “correct” description of the maternal blood circulation we identified the clearance exchange of chemical from the placenta to the fetus as the most sensitive factor. It remains unclear to what degree knowledge about the most essential compound-specific elements for PBK modelling (hepatic clearance, plasma protein binding and renal clearance) would have improved the accuracy of the parameters at any given model structure. These parameters are usually estimated from in vitro studies or predictive in silico models, but these tools were not available (progress has been made on developing QSARs and QSPRs for kinetic parameters in humans, but not rodents) and consequently, we were limited to a visual “best-fitting” approach for the ADME parameters. Nevertheless, the information from the RAR reports for the nine pesticides was considered sufficient for accomplishing our model aim, i.e. to determine by mathematical simulation the most likely intake dose (or dose range) that is expected to result in fetal concentrations associated with in vitro activity (reverse dosimetry). In light of the limited data availability it is obvious that it was only feasible to develop a simple PBK model with a rather limited flexibility, and as such the chemical domain to which this model applies is certainly limited.

5.2.2 Model structure and parameters

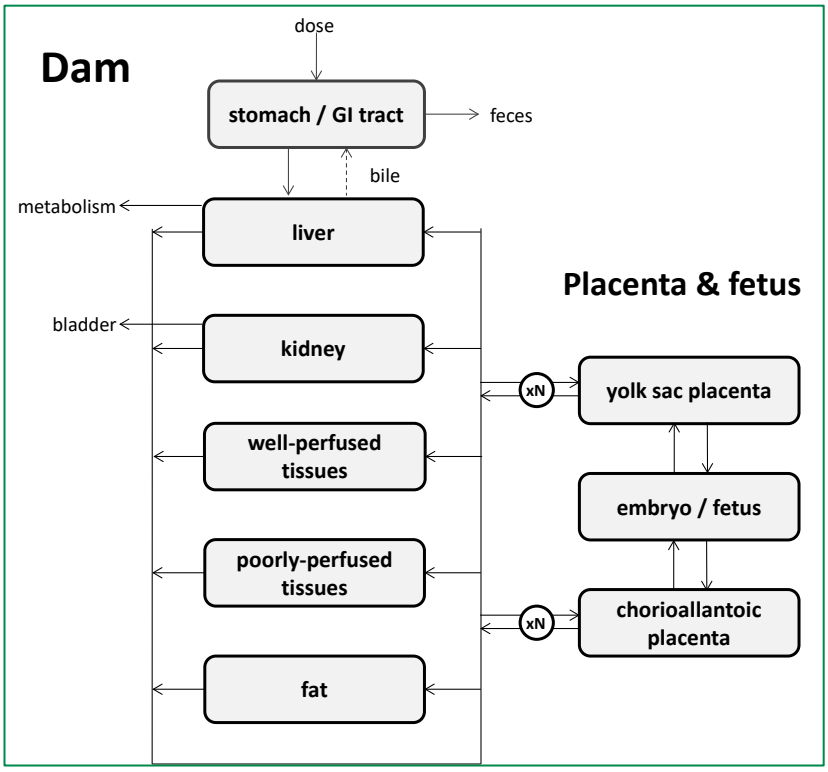

FIGURE. 2:. The PBK model structure including a fetal compartment

The PBK model structure is shown in fig. 2.

As we modelled the whole fetus rather than individual tissues, any simulation outcomes at the end of gestation should be considered with caution, as tissue-specific differences in fetal levels are more prominent and a total “average” burden might not be the right descriptor anymore. Fetal plasma levels were measured at GD21, but simulations optimized and conducted for the time between GD15 and GD 18, which should be taken into consideration when comparing the simulated and measured fetal levels.

The blood/plasma ratio was set to 0.55 (i.e. 1 - hematocrit) which assumes that the pesticides did not penetrate blood cells but were only restricted to the plasma. This should be considered a worst-case assumption, as the true compound-specific ratios are probably slightly higher. If instead a two-times higher ratio was used in the model simulation, the resulting average fetal concentrations were lower by only 1-5%.Therefore we consider this model factor as less sensitive for the simulation of fetal levels at given model structure.
As most critical parameter in terms of model uncertainty we identified the transfer between both placenta structures and the fetus. Here we followed the common assumption of a passive diffusion and used first-order clearance rates to describe the bi-directional placental transfer process (from mother to fetus and fetus to mother). Due to missing data we did not consider any elimination processes in the fetus, and therefore the function of amniotic fluid and its relationship to fetal levels were not included in the model structure. Here we see an urgent need for model improvement, which requires not only better knowledge about the placenta and its kinetic role for the transfer to the fetus, but also a corresponding in vitro or in silico data support.
The simple first-order kinetic assumption, together with no time delay integration, are mainly responsible for the artificial maximum peaks which we observed in the simulated time course pattern shortly after the intake dose, and therefore should not be used for any comparison to real measurements. For some pesticides (e.g. fenhexamid) the available in vivo data provided only very little kinetic details for the determination of a unique set of ADME model parameters and

as a consequence other combinations of similarly well-suited ADME model parameters would have achieved a similar good agreement between simulated and reported measurements. Although we tried as many as possible combinations in estimating the “best fitting” model parameters, we cannot rule out completely that we might have overlooked a better suited combination.

A description of the PBK model structure, with its various compartments, can be found in Appendix 2, together with a list of all the model parameters used.

5.2.3 Model calibration and verification

Prior to simulating the fetal levels for the selected pesticides we tested and optimised the model structure in two different ways:

1. The study by You et al (1999) on the trans-placental and lactational transfer of p,p’-DDE in rats was used to establish the generic PBK model structure (Fig 2). In this study fetal levels were measured at different time points after repeated dosing, and with our model simplifications we were able to recapitulate their observations within an error margin of ± 30%.
2. Three compounds were selected for further PBK modelling (procymidone, vinclozolin and prochloraz), all AR antagonists with in rodents well-characterized dose-response relationships for demasculinizing effects typical of anti-androgens. We used the reported LOAELs for reduced AGD as intake dose in our PBK model and compared the simulated fetal concentrations with in vitro concentrations reported for anti-androgenic activity. In all three cases we found an acceptable agreement, with simulations in the range of reported EC_{20} to EC_{80} values.

Plasma levels of the dams and fetuses measured in this project were not used for further model calibration, mainly because (1) they were taken at GD21, and (2) they did not provide sufficient information that would suggest what elements of the model structure (or model parameters) to change.
Results of the PBK model simulations are shown in Figure 3 for a range of repeated intake doses of dimethomorph, cyprodinil, fludioxonil, λ-cyhalothrin, quinoxyfen, fenhexamid, pyrimethanil, imazalil and o-phenylphenol (red shaded areas).
The range of simulated fetal concentrations refers to different assumptions about the bi-directional transfer between placenta and fetus, with the higher value assuming a higher transfer from the placenta into the fetus and a lower value a higher transfer from the fetus back to the placenta. The first setup would better represent a potential accumulation in the fetus, whereas a higher rate back to the fetus better simulate an (undefined) elimination from the fetus. The latter is probably more close to the biological data, as indicated by the lower levels detected in amniotic fluids compared to the fetus (see results section 5.5)..

5.3 Selection of pesticides and doses for the in vivo studies

Doses for in vivo testing were selected based on the integrated in vitro and PBK-modelling outcome as well as from known information on maternal or pup toxicity from prenatal toxicity, or 1 or 2 generation reproductive toxicity studies from EFSA’s DARs. Only intake doses below the NOAEL were assumed to produce no maternal toxicity and were favoured as potentially relevant for the in vivo testing. We used low effect concentrations for in vitro anti-androgenic activity (IC_{20}) and testosterone inhibition (LOEC) as target ranges in the fetus. The corresponding intake doses were calculated by using the PBK model and were expected to produce adverse effects on male reproductive health in rats. The overlap between active in vitro concentrations and low in vivo doses was apparent over a large dose range for fludioxonil, dimethomorph, cyprodinil, imazalil, and fenhexamid, and in vivo doses were chosen within these ranges.

The results showed that active in vitro concentrations (for either AR antagonism and/or testosterone inhibition) for all the pesticides except λ-cyhalothrin and quinoxyfen were in the range of the fetal levels predicted as resulting from intake doses below maternal toxicity (Fig. 3).

We started the prioritization process of possible candidates for further in vivo investigation by identifying those pesticides that we considered least likely to produce in vivo responses at non-toxic dose ranges. First, azinophos-methyl and pirimiphos-methyl were excluded due to their weak or non-detectable in vitro activity and a lack of reported kinetic in vivo data necessary for a meaningful PBK modelling (for that reason results on both of these are not shown in the report). Next, λ-cyhalothrin and quinoxyphen were excluded on the basis of kinetic considerations. We judged the doses for these two pesticides that would be required to attain fetal concentrations in the range of in vitro activity as far too high, and well within the dose range of maternal toxicity. Similar reasoning applied to pyrimethanil and fenhexamide, and these two pesticides were also excluded. Thus, out of an initial selection of 11 pesticides, we were left with 5 substances, and this selection was narrowed down further to arrive at a set for in vivo testing, by using the following ranking criteria:

The **1st priority** was fludioxonil. Both testosterone inhibition and AR antagonism were estimated to be active at fetal concentrations resulting from dose levels well below the reported NOAEL. Furthermore, the LOAEL of 210 mg/kg was derived from reductions in body weight in dams and pups in previous 2 generation studies - a dose which is close to the selected top dose in this study but well above the middle and low doses. Thus, a reasonable margin between male reproductive health effects and general toxicity could be expected.

As the **2nd priority** we suggested dimethomorph or o-phenylphenol. These pesticides were characterized by being primarily AR antagonists with only minor effects on testosterone synthesis. The predicted dose required to block AR in the fetus is obtained at a dose well below NOAEL. The LOAELs for these pesticides are based on effects on reduced maternal and pup body weight. Dimethomorph has previously shown indications of an anti-androgenic mode of action in vivo (prostate changes, increased testis weight and Leydig cell tumors in long term studies). Effective doses of dimethomorph are predicted to be lower than for o-phenylphenol and therefore we selected this pesticide.

As the **3rd priority** we suggested cyprodinil or imazalil. Both pesticides are mainly affecting testosterone synthesis and to a lesser extent blocking of the AR. The intake dose needed to achieve critical concentrations in the fetus that are expected to inhibit testosterone is predicted to be well below the NOAEL. For obtaining AR antagonism in the fetus, an intake dose above the NOAEL is needed. For cyprodinil the LOAEL of 100 mg/kg is based on reductions in maternal body weight and food consumption during gestation and reduced pup body weight during lactation in a rat 2 generation study. In a rat developmental study, cyprodinil had no effects at 200 mg/kg, but reduced maternal and pup body weight at 1000 mg/kg bw – both doses that are well below the selected top dose in this study. For imazalil the LOAEL of 80 mg/kg is for pup death, and selecting imazalil for experimental studies was therefore associated with a higher risk of non-target adverse effects than for cyprodinil.

Finally, three pesticides were selected for the developmental toxicity study: fludioxonil, dimethomorph and cyprodinil (highlighted in the Table 3). All fulfilled the requirements of being widely used on the European market, being potent anti-androgens in vitro and with a kinetic profile that favors intake doses which might result into critical target levels in the fetus without causing any maternal toxicity. The selected doses were 20, 60 and 180 mg/kg for fludioxonil and cyprodinil and 6.7, 20 and 60 mg/kg for dimethomorph.

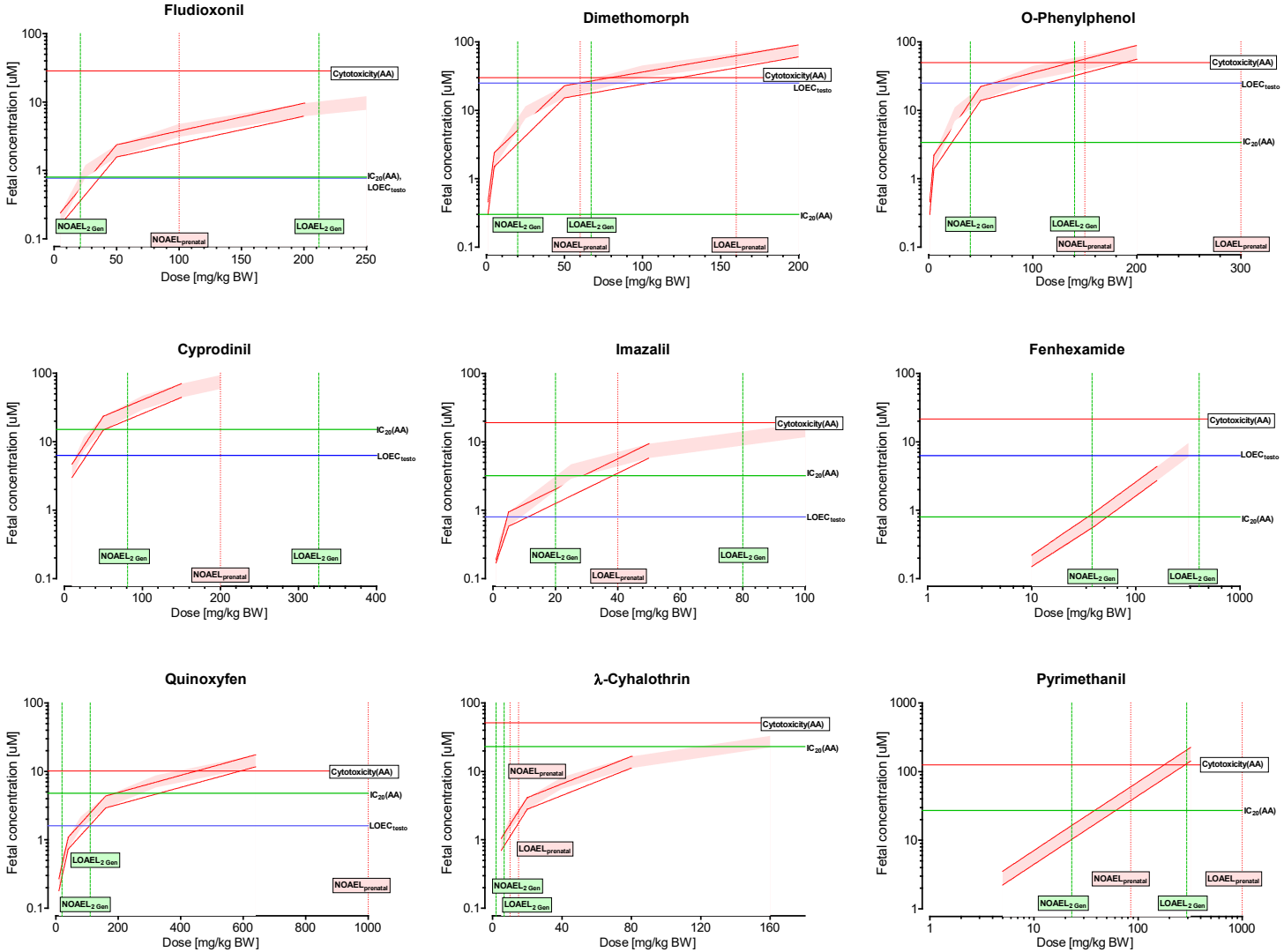

FIGURE. 3: Simulated fetal concentrations of dimethomorph, cyprodinil, fludioxonil, λ-cyhalothrin, quinoxyfen, fenhexamid, pyrimeethanil, imazalil and O-phenylphenol in response to repeated maternal doses (GD7 - GD21, red shaded areas) derived by PBK modelling. Horizontal red lines indicate concentrations associated with in vitro activity (cytoxicity, AR antagonism (AA), testosterone inhibition), dashed vertical lines indicate intake doses associated with *in vivo* responses (NOAELs and LOAELs reported for maternal and reproductive toxicity, for λ-cyhalothrin the NOAEL and LOAEL values are chosen from studies on cyhalothrin).

5.4 In vivo toxicity study – effects on AGD

The first objective of the in vivo study was to investigate if the pesticide levels predicted by the PBK models could be detected in the fetuses and whether the tested compounds would show primarily reductions in AGD and secondarily effects on other markers of antiandrogenic action. The selected pesticides have not previously been investigated for effects on sensitive endpoints for endocrine disruption such as AGD and the DARs have concluded that none of them were reproductive toxicants.

No signs of maternal toxicity during gestation and lactation were observed for any of the three compounds (Table 5). Only at highest dose of fludioxonil a significant 23% decrease in maternal weight gain was seen from gestation day (GD) 7 to 21 ($p=0.03$), which most likely was caused by a slightly reduced mean litter size. No changes on maternal weight gain GD 7 to PND 1 were detected, and therefore we conclude that no clear indications for maternal toxicity are present in the data. Furthermore, no significant effects on gestation length, post implantation loss, perinatal loss or body weight gain in the offspring were seen (Table 5). The number of non-pregnant dams was relatively equal among the exposure and control groups, with group sizes of 6-10 live litters per group (Table 5). None of the compounds significantly affected offspring birth weights.

All three pesticides caused significant reductions of 5-7% in male AGD at at least one dose. For fludioxonil and cyprodinil, significant reductions in male AGDs were seen at the mid dose of 60 mg/kg ($p=0.0124$ and $p=0.0017$, respectively). For dimethomorph, a significant AGD reduction was seen at the low 6.7 mg/kg and mid 20 mg/kg dose ($p=0.021$ and $p=0.005$), but not at the high dose ($p=0.96$) (Fig. 4). All three pesticides also caused significant reductions in female AGDs (Table 5). The effects on female AGD were generally observed in the same dose groups that were significant for male AGD.

No statistically significant effect on nipple retention (NR) at PND 14 was seen in the male pups exposed to either of the dose levels (Fig. 4), although a few dosed animals appeared with more nipples than controls.

The weights of various male reproductive organs were assessed on PND17 and no significant effects were seen, although a tendency towards a reduced prostate weight was observed for fludioxonil and cyprodinil (Table A3-4 in Appendix 5).

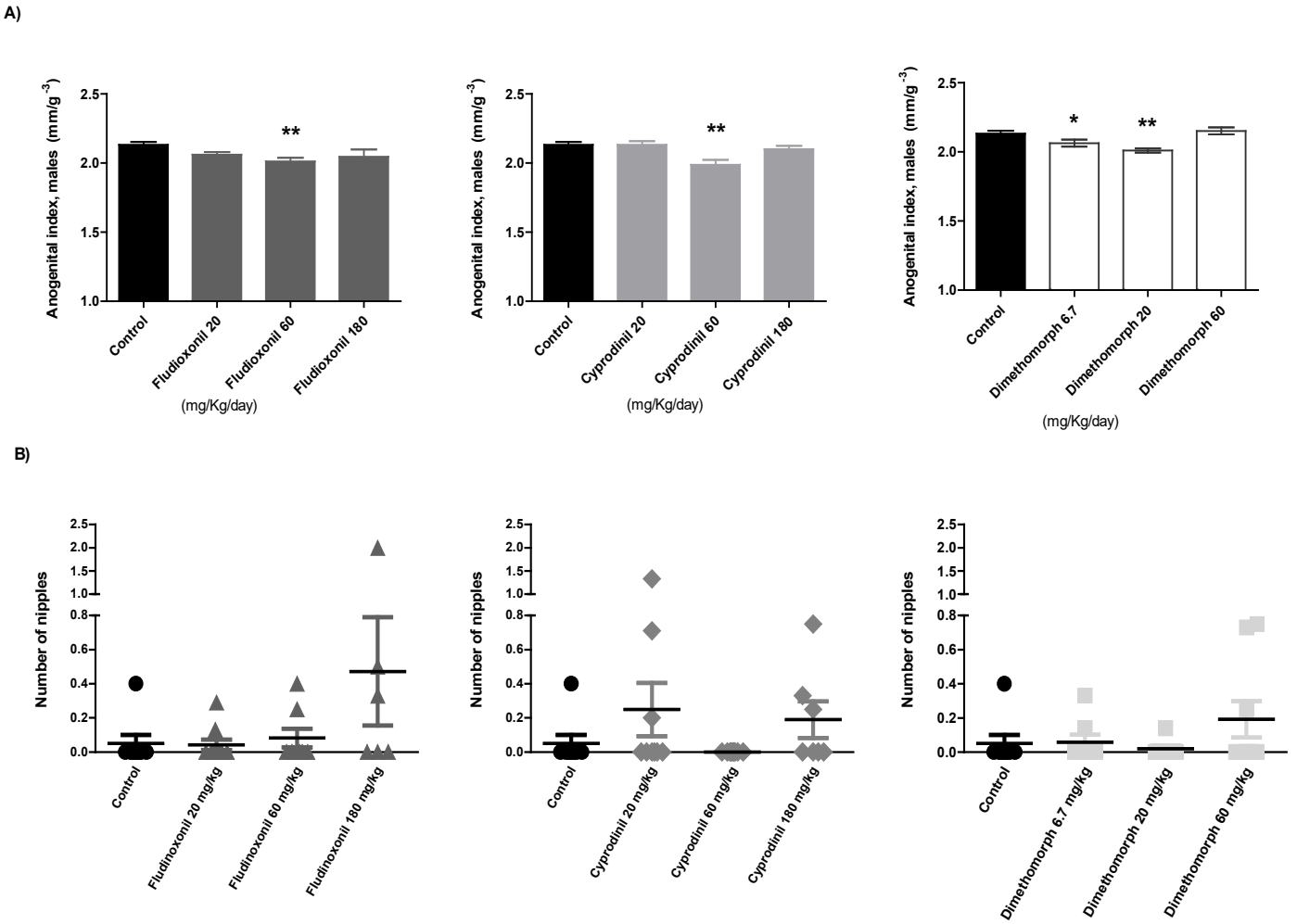

Fig 4. Effect on A) male anogenital distance (AGD) at birth and B) nipple retention pup day (PD) 14 after exposure to fludioxonil, cyprodinil (20, 60 or 180 mg/kg bw/day) or dimethomorph (6.7, 20 or 60 mg/kg bw/day). The data is presented as mean ± SEM. * $p<0.05$ and ** $p<0.01$ for Dunnett comparison with control group.

Table 5: Effects on dams and offspring

	1: Control	2: Flu-20	3: Flu-60	4: Flu-180	5: Cy-20	6: Cy-60	7: Cy-180	8: Dim-6.7	9: Dim-20	10: Dim-60
No. of dams (litters)	13+1(8)	10(10)	10+2(8)	10(6)	10(9)	10+2(9)	10(7)	10(8)	10+2(7)	10(9)
Dam bw gain,GD7-21 #	92.5 ± 9.5	92.7 ± 15.8	84.8 ± 9.7	**71.3 ± 20.7***	97.7 ± 8.3	78.7 ± 21.4	81.9 ± 11.0	96.6 ± 21.2	85.8 ± 17.7	100.4 ± 8.3
Dam bw gain, GD7- PD 1	19.8 ± 10.7	23.8 ± 10.5	16.8 ± 8.1	17.5 ± 14.9	23.5 ± 10.4	29.6 ± 5.8	21.8 ± 9.5	23.5 ± 9.8	21.2 ± 6.7	20.1 ± 9.8
Dam bw gain PD 1-14	33.34 ± 12.8	33.1 ± 12.7	38.1 ± 10.8	23.2 ± 15.5	32.4 ± 11.4	22.8 ± 13.7	18.9 ± 15.6	32.3 ± 11.3	31.4 ± 9.8	37.0 ± 13.8
Gestation lenght (days)	23.0 ± 0.0	23.0 ± 0.0	23.0 ± 0.0	22.8 ± 0.75	22.9 ± 0.17	22.9 ± 0.53	23.0 ± 0.0	22.8 ± 0.46	23.1 ± 0.38	23.0 ± 0.0
% postimplantation loss	21.5 ± 45.5	8.4 ± 10.2	15.9 ± 32.1	31.4 ± 36.0	17.1 ± 31.0	18.7 ± 33.1	7.7 ± 8.5	7.2 ± 7.8	19.5 ± 33.7	6.9 ± 8.3
% perinatal loss	22.1 ± 41.3	11.5 ± 11.9	18.7 ± 32.5	32.3 ± 35.6	18.0 ± 31.1	18.7 ± 33.1	7.7 ± 8.5	8.0 ± 8.7	20.8 ± 33.0	8.6 ± 8.7
Litter size	11.4 ± 3.2	10.5 ± 2.4	11.0 ± 1.3	8.2 ± 5.0	11.7 ± 2.2	7.1 ± 4.0	10.0 ± 2.6	11.4 ± 3.7	9.7 ± 3.7	12.2 ± 2.0
% postnatal deaths	0.8 ± 2.4	3.1 ± 9.7	3.4 ± 9.6	1.1 ± 2.7	1.2 ± 3.7	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 2.5	1.4 ± 3.8	2.6 ± 4.1
%males	43.2 ± 10.3	54.8 ± 15.6	47.2 ± 14.5	63.0 ± 20.8	48.2 ± 13.4	58.9 ± 23.1	46.5 ± 19.4	57.0 ± 16.7	56.6 ± 21.7	49.7 ± 17.6
Offspring										
Male birth weight	6.54 ± 0.6	6.48 ± 0.4	6.58 ± 0.4	6.51 ± 0.6	6.28 ± 0.4	6.68 ± 0.4	6.46 ± 0.3	6.29 ± 0.8	6.73 ± 0.5	6.35 ± 0.3
AGD males (units)	24.15 ± 1.1	23.26 ± 0.7	**22.83 ± 1.0***	23.11 ± 1.1	23.80 ± 0.9	**22.69 ± 1.3***	23.70 ± 0.8	**23.01 ± 0.5***	**22.98 ± 0.5****	24.15 ± 1.0
AGD males (mm)	3.99 ± 0.17	3.84 ± 0.12	**3.77 ± 0.16***	3.81 ± 0.18	3.93 ± 0.15	**3.74 ± 0.21***	3.91 ± 0.14	**3.80 ± 0.08***	**3.79 ± 0.08****	3.98 ± 0.16
AGI ma. (AGDmm/cu. root bw)	2.13 ± 0.06	2.06 ± 0.06	**2.01 ± 0.08***	2.05 ± 0.13	2.13 ± 0.08	**1.99 ± 0.10***	2.10 ± 0.06	2.06 ± 0.07	**2.01 ± 0.04****	2.15 ± 0.08
Nipple retention males	0.05 ± 0.14	0.04 ± 0.09	0.08 ± 0.16	0.47 ± 0.78	0.25 ± 0.47	0.0 ± 0.0	0.19 ± 0.28	0.06 ± 0.12	0.02 ± 0.05	0.19 ± 0.32
Female birth weight	6.26 ± 0.6	6.21 ± 0.5	6.18 ± 0.3	6.06 ± 0.5	5.88 ± 0.4	6.47 ± 0.5	6.08 ± 0.3	5.99 ± 0.7	6.17 ± 0.4	5.96 ± 0.4
AGD females (units)	13.74 ± 0.6	**13.04 ± 0.5***	**12.70 ± 0.6***	13.04 ± 0.6	13.14 ± 0.5	**12.89 ± 0.4***	13.21 ± 0.4	**12.87 ± 0.6****	**12.39 ± 0.3****	13.01 ± 0.6
AGD females (mm)	2.27 ± 0.09	**2.15 ± 0.09***	**2.10 ± 0.11***	2.15 ± 0.10	2.17 ± 0.08	**2.13 ± 0.07***	2.18 ± 0.07	**2.12 ± 0.10****	**2.04 ± 0.04****	2.15 ± 0.11
AGI fe.(AGDmm/cu. root bw)	1.23 ± 0.04	1.17 ± 0.04	**1.14 ± 0.05***	1.18 ± 0.06	1.20 ± 0.03	**1.14 ± 0.05***	1.20 ± 0.04	**1.17 ± 0.06***	**1.12 ± 0.03****	1.18 ± 0.05
Nipple retention females	12.29 ± 0.2	12.21 ± 0.2	12.13 ± 0.2	12.41 ± 0.6	12.35 ± 0.4	12.46 ± 0.7	12.31 ± 0.3	12.22 ± 0.2	12.34 ± 0.3	12.30 ± 0.3
Offsprping weight PD 6	12.58 ± 2.1	12.98 ± 1.3	13.20 ± 1.6	12.54 ± 1.9	12.39 ± 1.4	14.43 ± 1.6	12.72 ± 1.7	13.19 ± 2.3	13.10 ± 1.2	13.47 ± 2.1
Offsprping weight PD 14	26.81 ± 4.7	28.07 ± 3.3	28.41 ± 4.2	26.58 ± 4.1	26.61 ± 4.2	**33.21 ± 5.3***	27.34 ± 4.5	27.69 ± 5.8	28.25 ± 3.3	26.37 ± 2.9
Male weights PD 23	47.04 ± 8.2	48.66 ± 6.1	49.78 ± 6.9	47.97 ± 5.6	-	-	-	-	-	-

Dam BW gain was calculated for dams giving birth to viable litters (i.e. the dams having 100% postimplantation loss, and the dams used for GD21 section were excluded)
Data represents mean ± SD. Bold values are statistically significant (* p<0.05; ** p<0.01)

5.5 Analysis of parent pesticides and their metabolites

For the three pesticides tested in vivo, dimethomorph, cyprodinil and fludioxonil, the amniotic fluid and fetal plasma levels in controls and middle doses were analysed on GD21 (ca. 90 minutes after dosing) taken from two dams (Table 6). We measured the pesticide exposure of the foetuses 1½ hour after dosing, as maximum exposure levels are usually observed 1-2 hours after dosing for most fast-metabolized chemicals.The levels of parent pesticides and metabolites were quantified and semiquantified, respectively, in dams, fetuses and amniotic fluid using a LC-QTOF.
Generally, the pesticide plasma levels were similar in male and female fetuses. Also the metabolite profiles of all three pesticides were generally similar in plasma from dams, female and male fetuses (Table 6).
However, for fludioxonil and cyprodinil a larger fraction of the respective compound was detected to occur in metabolised form in the plasma from dams and female fetuses than in the plasma from male fetuses.
Several phase 2 metabolites of the oxidised fludioxinil were observed in both plasma and amniotic fluid in accordance with information given in the DAR. The primary metabolite of cyprodinil was an oxidised metabolite, and dimethomorph was only metabolised to a minor extent which may indicate that it takes longer than 90 min to have this compound metabolized.

In *Appendix 3* is shown representative chromatographic data from fludioxonil 60 mg/kg, cyprodinil 60 mg/kg and dimethomorph 20 mg/kg, in plasma from dams, amniotic fluid, and male fetal plasma. No pesticide residues could be detected in the control animals.

5.6 Comparison of measured pesticide levels and those predicted by PBK modelling

Table 6: Measured and simulated pesticide levels in maternal plasma, fetal plasma and amniotic fluid after repeated dosing of fludioxonil, cyprodinil, and dimethomorph (GD7 - GD21). Pesticides were measured on GD21 (ca. 90 min after dosing), simulations refer to plasma levels on GD21 (dams) and to average levels during GD15 - GD18 (fetuses). N=2 pregnant dams, fetal blood pooled for each litter for males and females, respectively.

		Fludioxonil 60 mg/kg	Cyprodinil 60 mg/kg	Dimethomorph 20 mg/kg
Maternal blood	Measured[&]	1.3 - 1.7 µM	4.1 - 11.0 µM	3.3 - 5.1 µM
	Simulated*	10 - 3.6 µM	32.8 - 10.2 µM	3.1 - 1.0 µM
Amniotic fluid	Measured[&]	0.6 - 1.3 µM	0.8 – 1.6 µM	0.8 µM
Male foetus	Measured[&]	2.3 - 4.3 µM	5.5 – 7.6 µM	1.3 – 1.4 µM
Female foetus	Measured[&]	2.0 – 5.0 µM	5.2 – 9.9 µM	1.3 – 1.3 µM
Foetuses	Simulated	3.3 - 5.4 µM	11.5 - 18.3 µM	1.0 - 1.5 µM
Ratio between measured & simulated fetal levels[#]		0.7 – 0.9	0.5 – 0.5	0.9 – 1.3

* corresponding to 2 - 6 hours after dosing
# the average of male and female measured values were used
& measured values from dam/litter 1 and dam/litter 2 are shown

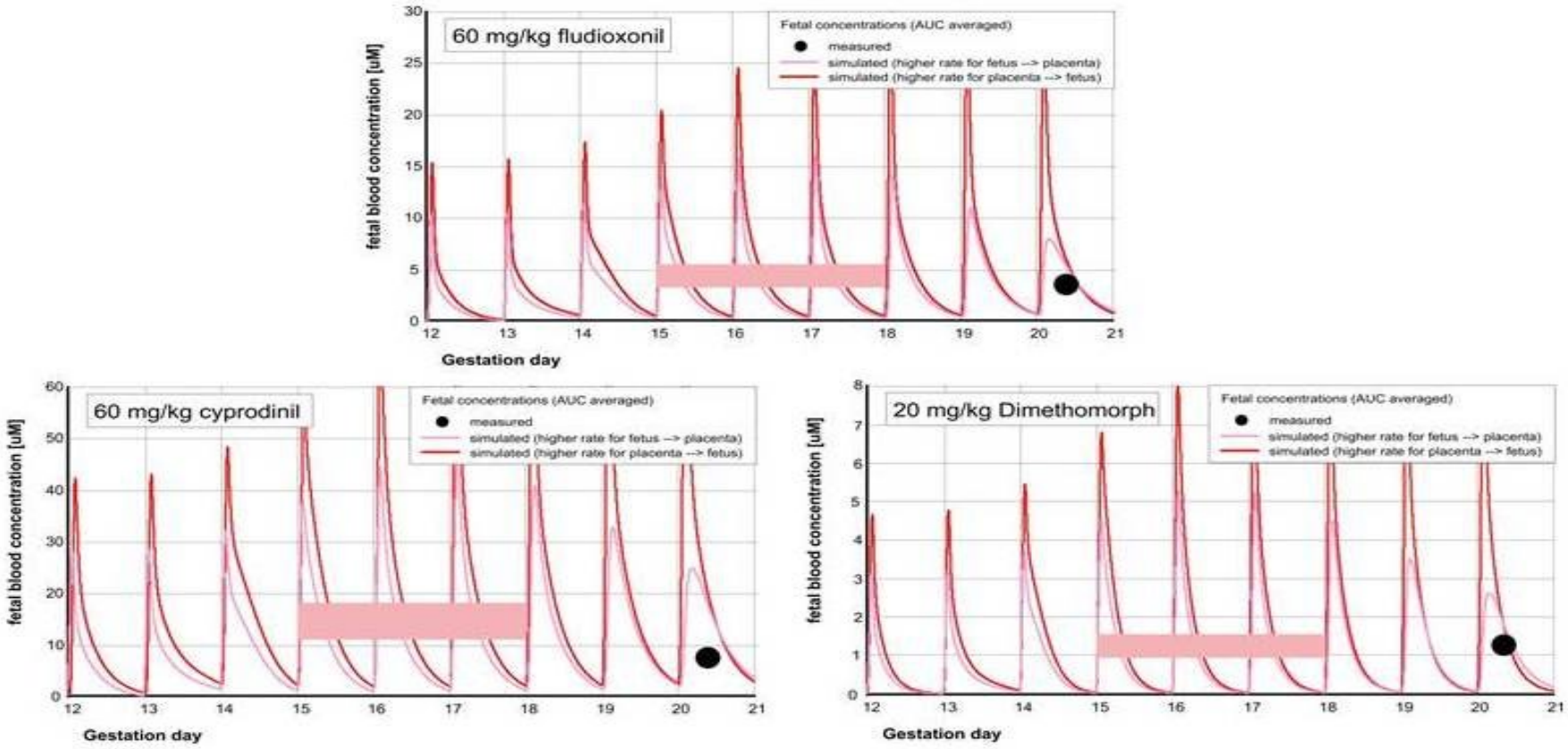

FIGURE. 5: Simulated time course of fetal concentrations of pesticides and their measured fetal levels at GD21 (black dot, N=2). Shaded area equals the range of average estimates (AUC per hour). Two profiles are depicted for two different model setups of the bi-directional placental transfer from mother to fetus and fetus to mother.

Figure 5 shows the simulated time course of pesticide concentrations in the fetal compartment at medium intake doses, together with the measured fetal levels at GD21 (black dot, N=2). The shaded area shows the range of average estimates (AUC per hour) for two different assumptions regarding the bi-directional placental transfer from mother to fetus and fetus to mother. The average plasma levels of fludioxonil and dimethomorph measured in the fetus agreed well with the concentration window simulated by the PBK model, and for cyprodinil we simulated levels approx. two-fold higher than measured (Table 6).

5.7 Correlation between in vitro activity at actual fetal levels and AGD effects

Based on the measured pesticide levels in the fetuses at the mid doses where significant effects on AGD occurred, we wanted to get an idea of the in vitro potency of the pesticides for each of the molecular mechanisms (Table 7). For cyprodinil the measured male fetal plasma concentration of ~6.6 µM corresponds to a concentration in vitro where a marked PG synthesis inhibition and a moderate testosterone synthesis inhibition can be expected (IC_{30}). The measured concentrations are too low to expect AR antagonism of cyprodinil.

For fludioxonil the measured male fetal plasma concentration of ~3.3 µM would result in a moderate AR antagonism (IC_{58}) and a weaker testosterone inhibition (IC_{37}) (Table 7).

For dimethomorph the measured male fetal plasma concentration of ~1.3 µM is expected to cause a marked AR antagonism (IC_{62}), whereas testosterone synthesis is not expected to be significantly affected (Fig 6 & Table 7).

These comparisons between in vitro activities and actual internal concentration at the target assume that serum concentrations in the rat fetus can be translated 1:1 into effective in vitro concentrations. However, this may be too simplistic as the albumin and protein levels in the in vitro cell culture media are different from those in the in vivo compartments, resulting in different free levels of active compounds. Therefore these comparisons should be considered with caution.

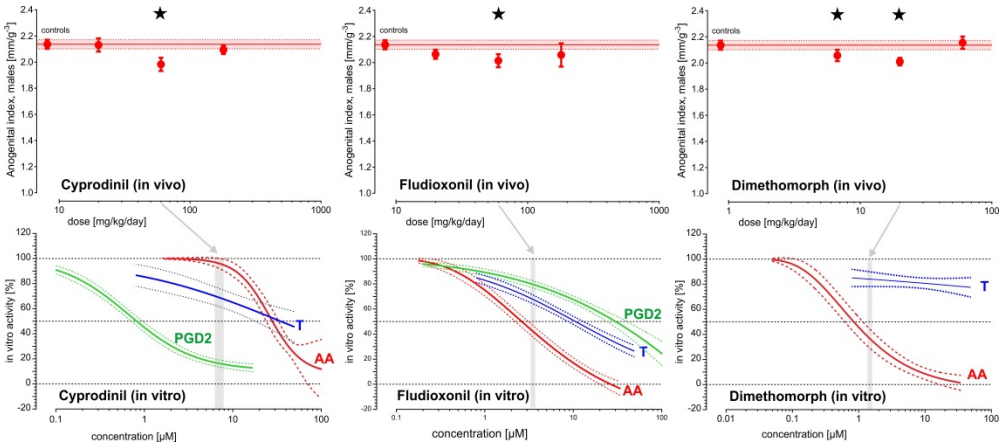

FIGURE. 6: The top figures show the effect on **anogenital distance (AGD) at birth** after exposure to fludioxonil, cyprodinil (20, 60 or 180 mg/kg bw/day) or dimethomorph (6.7, 20 or 60 mg/kg bw/day). The bottom figures show the concentration-response curves for **AR antagonism** (AA) (red line), **testosterone inhibition** (T) (blue line), as well as for **prostaglandin D2 (PGD2) suppression** (green curve). The vertical gray line indicates the measured male fetal plasma concentration of the respective pesticide after exposure of dams to cyprodinil 60 mg/kg, fludioxonil 60 mg/kg, or dimethomorph 20 mg/kg, respectively (gray arrow). The AR and PGD2 data are from Orton et al. (2011) and Kugathas et al., (2016) respectively.

Table 7: Comparison of measured fetal pesticide exposure and *in vitro* effect concentrations
Measured pesticide levels in male foetal plasma for fludioxonil and cyprodinil (60 mg/kg) and dimethomorph (20 mg/kg) along with corresponding IC values for *in vitro* AR antagonism and testosterone inhibition. IC_{50} from AR antagonism and testosterone inhibition *in vitro* assays are shown as well.

	Fludioxonil 60 mg/kg	Cyprodinil 60 mg/kg	Dimethomorph 20 mg/kg
Plasma levels in male foetuses	3.3 µM	6.6 µM	1.3 µM
IC value at the measured male plasma level [#]	58 / 37	4 / 51	62 / -
IC_{50} for AR antagonism	2.6 µM	28 µM	0.9 µM
IC_{50} for Testosterone inhibition	10.2 µM	24 µM	ND

The plasma levels represents the mean from two male foetuses,
[#] The listed IC values are for AR antagomism/ testosterone inhibition
ND: could not be determined

5.8 Long-term health effects of fludioxonil

No changes in reproductive organ weights were evident at 3 or 11 months of age in male offspring exposed to fludioxonil (Appendix 5 Table A3-4). Since fludioxonil was expected to have anti-androgenic effects, we nevertheless looked for more subtle molecular or cellular phenotypes in the androgen-sensitive tissues testes and prostate, as described in the following sections.

5.8.1 Effects on plasma hormone levels

Blood hormone levels were measured in control and fludioxonil-exposed animals at 3 months of age (Fig. 7). Although levels of testosterone, lutenizing hormone (LH) and sex hormone-binding globulin (SHBG) were judged by statistics as non-different between the control and treatment groups, testosterone and LH data indicate negative trends towards higher fludioxonil exposures.

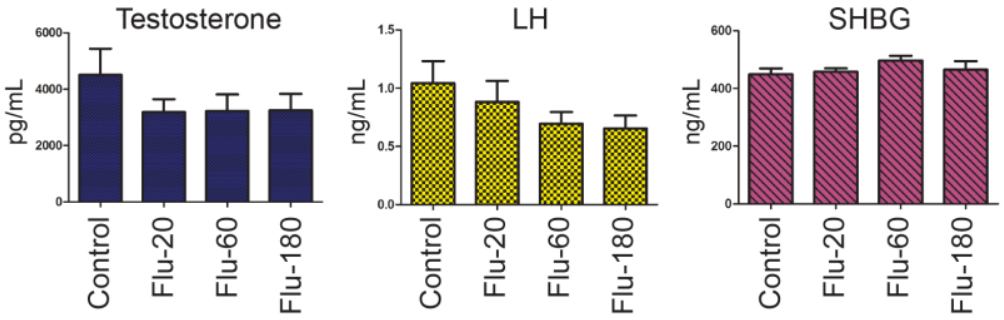

FIGURE. 7: Serum hormone levels in 3 months old rats after early-life exposure to fludioxonil. Rats exposed to fludioxonil from GD7 to PND 22 showed a trend towards reduced blood testosterone and LH levels at 14 weeks of age. SHBG was unaffected. Data represents mean ± SEM (n=10-20).

5.8.2 Effects on testis gene expression

From the H295R steroidogenesis data (Section 5.1 and Appendix 4) it is clear that fludioxonil can suppress testosterone synthesis. Also, in adult male offspring from animals exposed to fludioxonil during fetal life, there was a clear tendency towards reduced testosterone levels. Therefore we analyzed the relative transcript abundance of selected cell-specific marker genes of the adult testes from fludioxonil exposed animals (Fig. 8). These genes include many key factors involved in Leydig cell steroidogenesis and potential targets for pesticide disruption, but also more general marker genes for Leydig (e.g. *Nr5a1*, *Insl3*), Sertoli (e.g. *Sox9*) and germ (e.g. *Ddx4*) cells to simultaneously monitor any major changes in overall changes in cell ratios within testes. In the 3 month old testes, we observed that *Star* expression was reduced by approximately 40% in the high dose group. This indicates that cholesterol transport is affected. No significant change in transcript abundance of the germ cell (*Ddx4*), Sertoli cell (*Sox9*) or somatic cell (*Ar*, *Nr5a1*) specific genes, nor for the Leydig cell specific genes *Insl3*, *Lhcgr*, *Cyp11a1*, *Cyp17a1* and *Hsd3b1*was observed. No change of *Dlk1* was seen.Although it cannot be determined without histological assessment of the testis, including additional time points, the stable expression (in relative terms) of marker genes suggests that the overall cellularity of the testes were maintained in all the exposure groups (Almstrup et al 2004) such that a reduction in Leydig cell numbers is unlikely the cause of reduced testosterone levels. In the 11 month old testes a very slight reduction in expression of *Cyp11a1* and *Hsd3b1* was observed in the mid-dose group. The effect on *Star* expression was not noticeable at this age. Thus, these analyses indicated a few, minor alterations of testis development or function at the gene expression level.

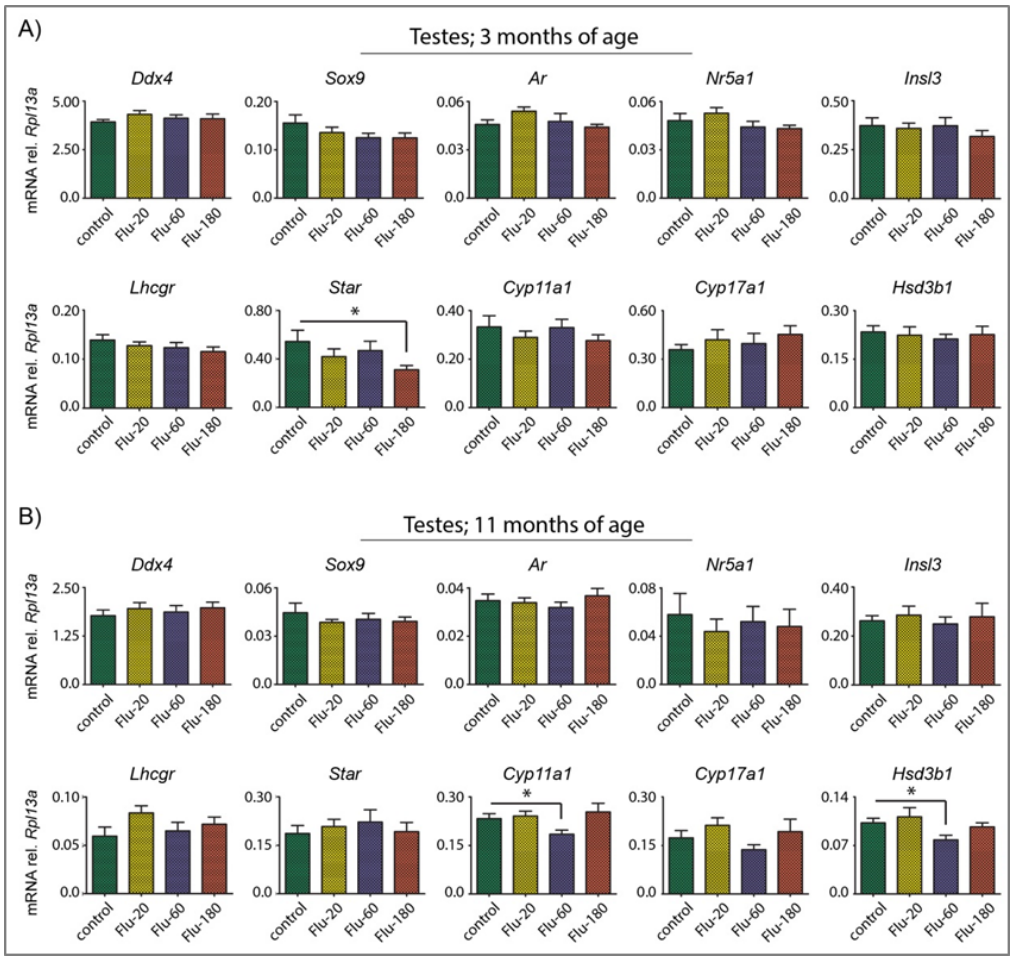

FIGURE. 8: Relative expression of cell-specific marker genes in adult rat testes after in utero exposure to fludioxonil. Expression profiling was carried out on testes from A) 3 month and B) 11 month old rats. The expression of the germ cell marker *Ddx4*, Sertoli cell marker *Sox9,* and somatic markers *Ar* and *Nr5a1* were relatively unchanged across all the exposure groups relative to control at both ages. Among the Leydig cell-specific genes, *Insl3*. *Lhcgr*, *Star*, *Cyp11a1*, *Cyp17a1* and *Hsd3b1),* only *Star* displayed a noticeable change in expression level in the 3-month old testes, and only in the high dose group. In the 11 month old testes, *Star* expression was no longer different between any groups, whereas *Cyp11a1* and *Hsd3b1* were slightly downregulated in the mid-dose group. No gene displayed statistically significant variance by ANOVA; statistical significance was observed by Student's t-test only, *p< 0.05. N = 10-12.

5.8.3 Effects on prostate gene expression, histology and epigenetics

Persistent effects on the prostate in animals have been reported after exposure to androgen-disrupting compounds (Isling et al, 2014). We therefore analyzed the prostate of adult male offspring that had been exposed to fludioxonil during early development. As an initial assessment, two ventral prostate samples from each exposure group and controls from 2 weeks and 3 months of age were analyzed using an 84-gene custom array to profile potential changes in expression of known factors implicated in various prostate pathologies, not least oncogenic transformation (Fig. 9). In the juvenile male rats, 2 weeks old, very few changes in gene expression were observed. In prostates from 3 month old rats, however, several genes were dysregulated in all of the dose groups relative to control animals.

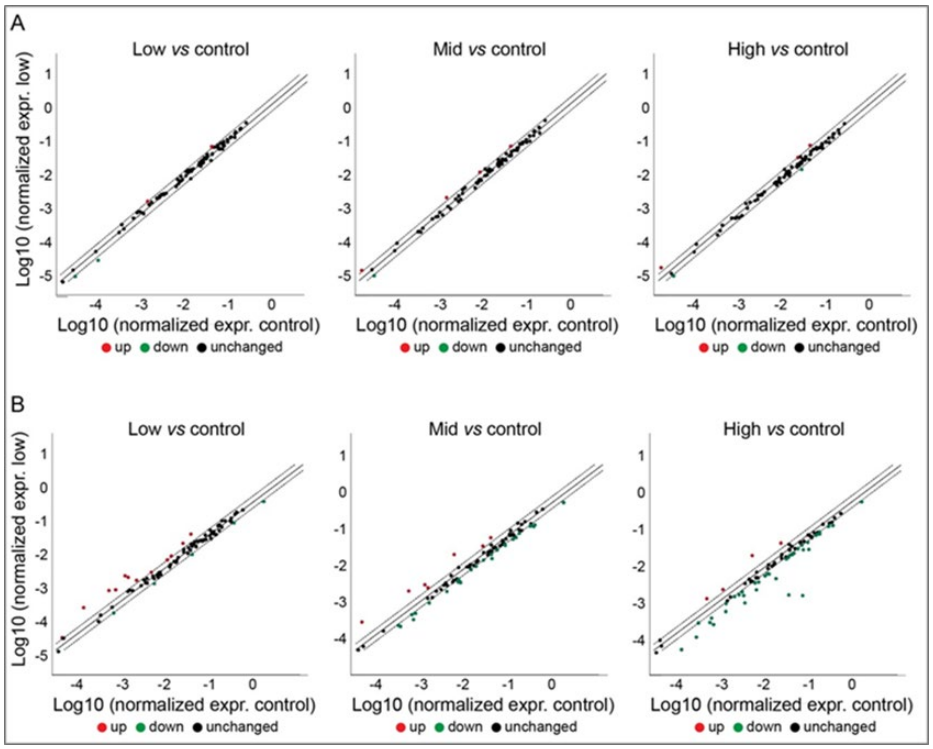

FIGURE. 9: Prostate 84-gene array on tissues obtained from fludioxonil-exposed male offspring. Scatter-plots of differentially expressed genes in prostate tissue from control versus exposed animals. A) Three dose groups versus control in 2 week old rats. B) Three dose groups versus control in 3 months old rats. Genes up-regulated more than 2-fold are highlighted in red and genes down-regulated more than 2-fold highlighted in green. Values were obtained from two biological replicates (n=2) of each group. Centre line demarcates mean expression of control samples; dotted lines demarcate 2-fold up- or down-regulation relative to control.

The genes that displayed changes in relative fold expression relative to control tissues represent diverse functions within prostate development and function, and have all been linked to prostate dysgenesis (Massie et al 2016). They include the key transcription factors *Ar*, *Nkx3.1*, *Daxx* and *Rarb*, the androgen-sensitive receptor *Egfr*, as well as genes involved in cell cycle and apoptosis such as *Ptgs2* and *Cdkn1a*. Therefore, a more in-depth analysis of the transcriptional levels of selected genes was conducted by RT-qPCR, now including all the biological replicates across the different exposure groups.

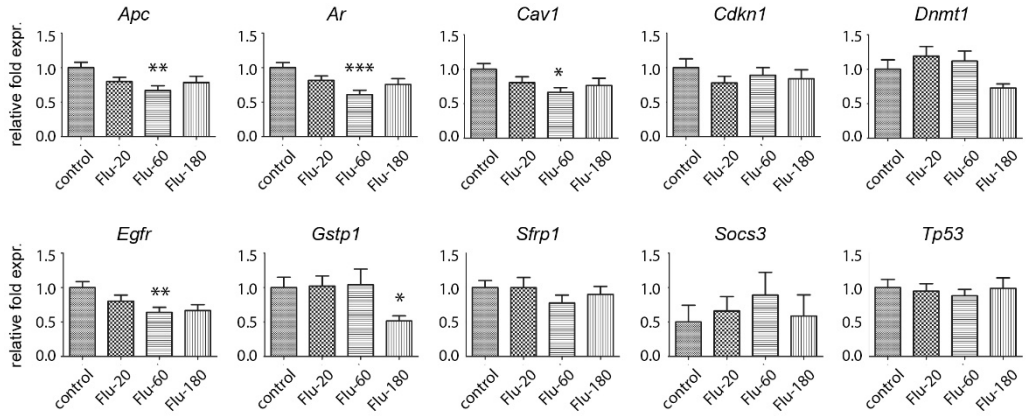

FIGURE. 10: Relative mRNA expression in prostates of 3 months old rats following early-life exposure to fludioxonil. Relative mRNA levels were determined for 10 genes; *Apc*, *Ar*, *Cav1*, *Cdkn1*, *Dnmt1*, *Egfr*, *Gstp1*, *Sfrp1*, *Socs3* and *Tp53*). Graphs represent relative mRNA levels with mean (± SEM) values of control group set to 1 (Comparative Ct-method; normalized with the geometric mean of *Hprt* and *Rpl13a*).Group sizes were; control (n

=14), low (n = 20), mid (n = 15), high (n = 10). Statistical significance (*p <0.05, **p <0.01, ***p <0.001) was determined by One-way ANOVA.

The RT-qPCR results (Fig. 10) validated the small gene array pilot screen (Fig. 9) which indicated that several key androgen-sensitive genes were dysregulated in the prostate of adult male rats, long after exposure to fludioxonil had ceased. Although the relative fold changes in transcript abundance were small at the group level, a few individual samples displayed relatively large changes, which could be consistent with alterations to promoter methylation statuses at the individual levels. However, to further substantiate a causative link between early exposure to fludioxonil and late-life changes in transcription by way of altered promoter methylation, larger samples sizes coupled with methylation sequencing is needed. However, the data do imply a persistent effect following early life exposure to this current use pesticide, which potentially can cause late-onset disease of the prostate tissue. Genes that were downregulated in the exposed animals include key regulatory factors such as *Ar*, *Apc* and *Egfr*, all of which have been shown to be dysregulated in transformed prostate tissue. It remains uncertain to what degree some of these factors are involved in inducing adverse effects, but they nevertheless point towards disrupted tissue homeostasis. Therefore, the prostate tissue from these animals was further analyzed for any histopathological signs.

Histological examination of ventral and dorsolateral prostate at 3 months of age showed marked inflammation in several animals. A high number of males, including three controls, showed marked interstitial inflammation and this was associated with epithelial inflammation and atypical hyperplasia of epithelial cells (Fig. 11B). Although high incidences of these lesions were seen in ventral prostates of fludioxonil-exposed males, this was not significantly different from controls (Fig. 11 C and D; Appendix 5.2). In all three affected control males, the interstitial inflammation and reactive hyperplasia was marked, whereas the severity of effects differed more in the fludioxonil group.
At 11 months of age, no clear histological effects could be detected in ventral or dorsolateral prostate. Generally, low scores for atypical epithelial hyperplasia was seen compared to the examination at 3 months of age, and no interstitial inflammation was observed. Morphometric analyses showed no differences in the distribution of epithelial, luminal and stromal areas (for further details see *Appendix 5.2*).

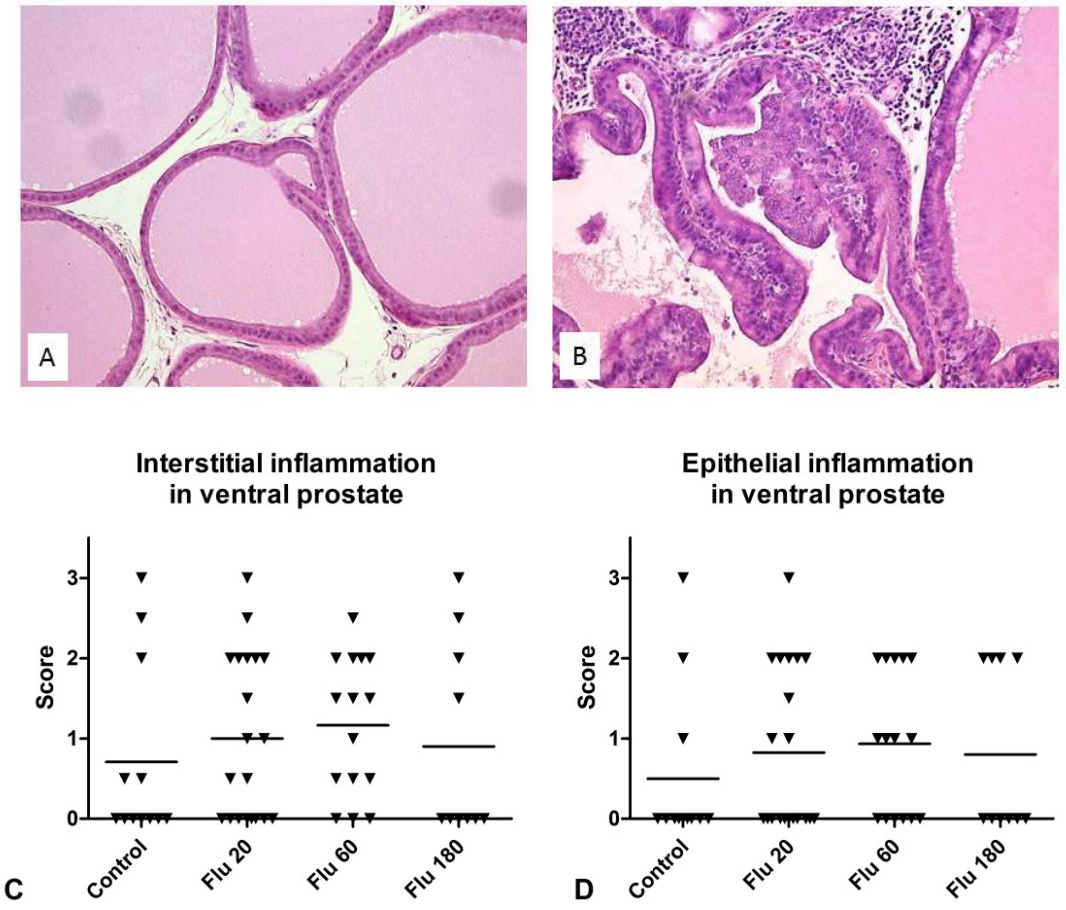

FIGURE 11: Interstitial inflammation associated with atypical hyperplasia of epithelial cells in ventral prostate (B) was observed in control as well as fludioxonil-treated animals at three months of age. A-B: H&E stained sections from control animals (original magnification 20x). C-D: plot of scores for histological findings in ventral prostate at three months of age (individual values; no significant differences between dose groups).

Although the histopathological assessment failed to find any persistent signs of disease states, the promoter methylation status was examined for four of the genes that were shown to be downregulated in the fludioxonil exposed offspring. This was done since hypermethylation of regulatory promoter sequences generally causes reduced expression of the gene. Unmethylated promoters are more readily available for regulatory proteins and thus more often seen in actively transcribed genes. In other words, promoter methylation is a way in which gene expression is regulated. The four genes that were downregulated in the prostate of fludioxonil-exposed animals (*Apc, Ar, Cav1* and *Gstp1*) are androgen-sensitive factors that have been shown to be involved in oncogenic transformation of prostate tissues and can thus help explain late-onset disease following insults that have occurred much earlier in life. Prostates from four rats in each exposure group at 3 months of age were screened using methylation specific PCR protocols targeting known CpG sites (Fig. 12). Significant inter-specimen variability of the methylation status was observed within all groups, including control animals, and the general trend was that most promoter regions were methylated rather than unmethylated at this developmental stage. The only promoter that showed abnormal hypermethylation as an average across the four samples was *Gstp1*, but only in the high dose group. This could suggest that fludioxonil exposure can cause abnormal methylation of this gene promoter, which in turn may result in downregulation of gene expression. The fact that the other genes, despite being

down-regulated at the mRNA level, did not show abnormal methylation status means that either promoter methylation is not the mechanism causing downregulation of expression, or that hypermethylation occurs at sites other than the proximal promoter region that were analyzed.

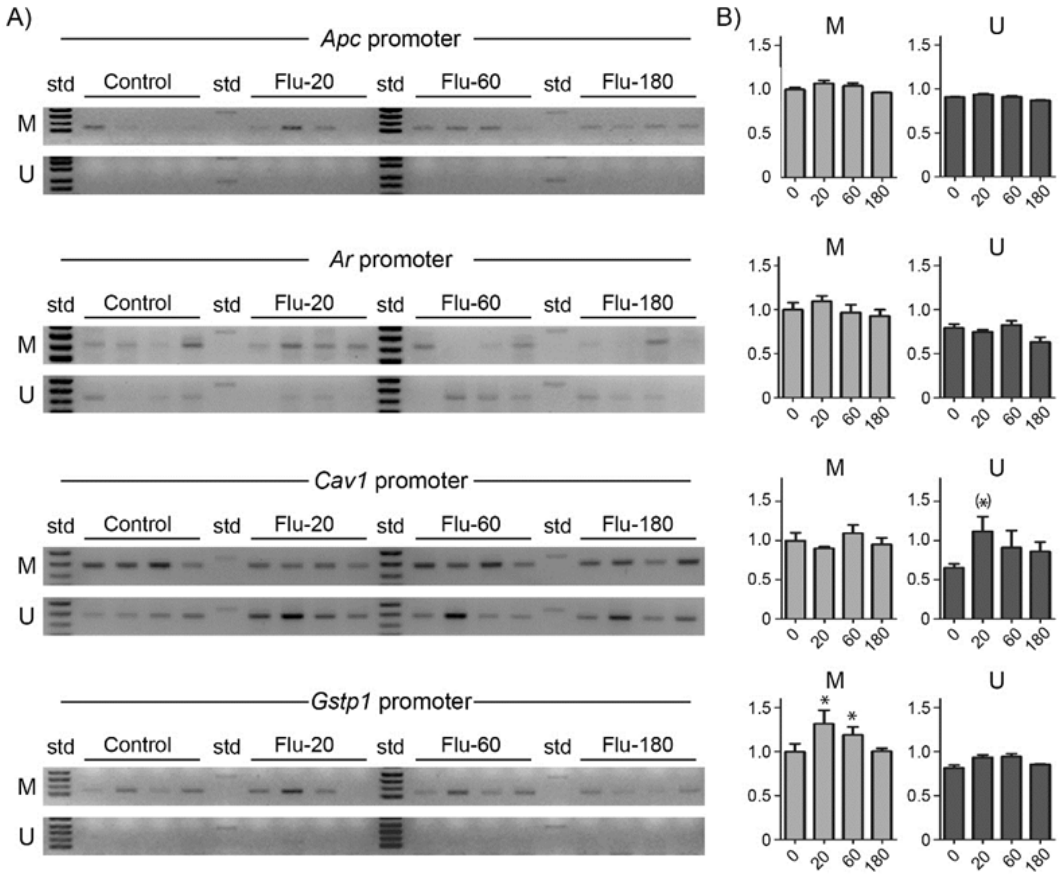

FIGURE. 12: Methylation status of the distal promoter of androgen-sensitive genes in prostates of 3 months old rats after early-life exposure to fludioxonil. Specific primers were designed around known CpG sites of the distal promoter (-1200 to +300 nt relative to TSS) and tested on bisulphide-converted genomic DNA extracted from ventral prostates. A) Four individual rats from each of the exposure groups (Flu-20, Flu-60, Flu-180) and controls, are represented with unique PCR bands going left to right. Each individual prostate are matched going from top to bottom. In general, the distal promoters were more frequently methylated. B) Average densitometry measurements of each band (mean ± SD; n=4) within each exposure group. Only the *Gstp1* promoter was, on average, more methylated after exposure to fludioxonil. std = molecular standard; M = methylated; U = unmethylated. Statistical significance, exposure group relative to control, was tested by unpaired two-tailed Student's t-test (*p <0.05).

6. Discussion

There is an urgent need for evaluating currently used pesticides for endocrine disrupting effects. However, animal testing is resource demanding and any development of methods that can reduce, avoid or replace the use of animals would be a big step forward. In this project we investigated the potential of using in vitro techniques together with PBK modelling as a tool for predicting possible in vivo anti-androgenic effects of early life exposure to pesticides. The primary aim was to develop a strategy for prioritizing pesticides for further in vivo testing by excluding those compounds which on evidence of all available information are unlikely to cause adverse anti-androgenic response in young offspring manifested as changes in primarily AGD and secondarily other markers of anti-androgenic action. Furthermore we were also interested in investigating the long-term consequences of fetal exposure to anti-androgenic pesticides.

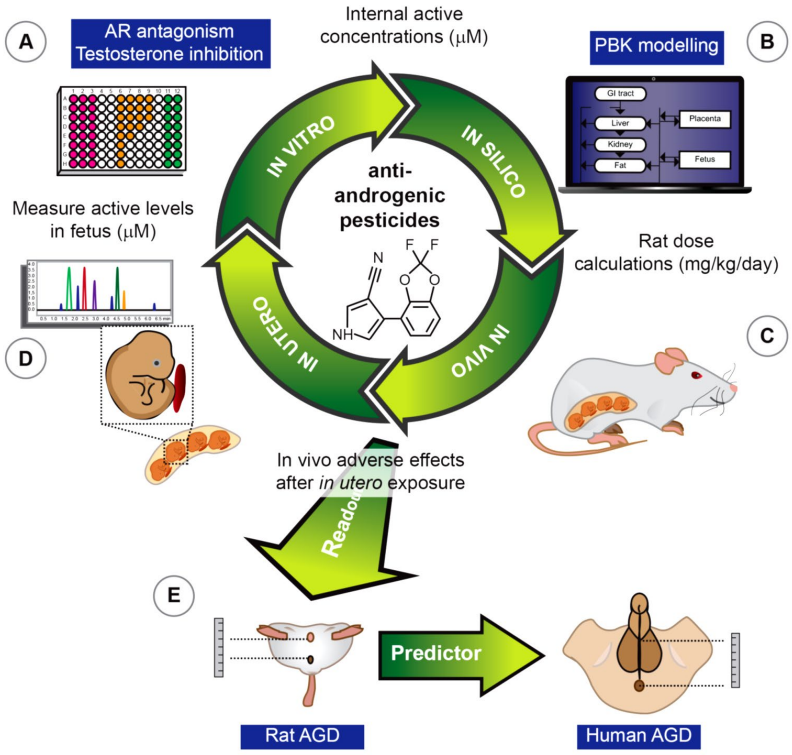

FIGURE.13: A graphical illustration of the integrated in vitro/PBK modelling approach for predicting AGD effects, including 'validation' studies of predicted exposures in rat fetuses.

In the following the main results will be discussed with regards to each of the working hypotheses.

6.1 A generic PBK model including the fetal compartment

Hypothesis 1: *It is possible to develop generic PBK models for antiandrogenic pesticides that are capable of simulating measured fetal concentrations in rats after repeated oral gavage*

Hypothesis 2: In vivo doses of anti-androgenic agents that will result in fetal concentrations associated with in vitro anti-androgenic activity can be predicted (reverse dosimetry).

In developing the PBK model we faced challenges in terms of the highly dynamic changes in the physiology of the dam during pregnancy, our limited understanding of blood transfer between the dam and the fetus as well as the model parameterization of all chemical-specific kinetic parameters. As a consequence we kept the model structure as simple as possible, with the main focus on simulating the blood circulation in the dam as close as possible to all reported kinetic data. In estimating the unknown transfer rates between the placenta structures and the fetus we made recourse to a range of likely values obtained from the literature. The average plasma levels of fludioxonil and dimethomorph measured in the fetus agreed well with the concentration window simulated by the PBK model, and for cyprodinil we simulated levels that were approximately two-fold higher than measured. Considering the data gaps and uncertainties that had to be bridged, this is an excellent performance of the PBK model. It would seem that a higher degree of model complexity is not required for the fetal compartment, at least not for the three selected pesticides.

Kinetic data have been reported for cyprodinil after a low dose (0.54 mg/kg) was administered by gavage to adult rats. Here concentrations were measured only within the first 17 hours after administration (0.25, 1.25, 11 and 17hrs, and we were forced to focuse the estimation of the ADME model parameters on the data measured after 11 and 17 hrs, as we considered the kinetic dynamics shortly after dosing as not relevant for our model structure and purpose. As the measurements suggest, we probably overestimated the plasma levels in systemic circulation, which could also explain why we predicted higher fetal levels than were measured. For dimethomorph we achieved a good agreement between simulated and measured maternal plasma levels, which probably explains why a similarly good agreement for the fetal compartment was observed. This highlights the importance of an accurate description of the systemic blood circulation for the estimation of fetal levels, if a misjudgment occurred here it is very likely that the corresponding fetal levels will be also be misjudged.

Probably most crucial for accurate prediction of the maternal blood circulation in non-pregnant rodents was the availability of in vivo kinetic data from the DAR reports, which ensured an estimation of the kinetic model parameters most likely similar to the values in our study. Also important was that we assumed that metabolites were not relevant for the model simulation, with the underlying assumption that the unmetabolised parent compound is responsible for anti-androgenic effects. Although this study provides no indications contradicting these suppositions, further confirmation on the basis of in silico tools (e.g. QSAR) or in vitro testing would certainly strengthen our assumption. If one (or more) metabolite is considered as potentially relevant and thus necessary for the model simulation, the same PBK model would have been extended with a second model layer referring to the metabolite without changing the fundamental structure of the model (i.e. using the same compartments). However, such a model extension would have required the estimation of kinetic model parameters for the metabolite for its distribution and elimination, something which is not available from published in vivo kinetic data in the DAR reports.

6.2 The in vivo anti-androgenic effects

Hypothesis 3: *Pesticides with high in vitro anti-androgenic activity and expected to reach the fetus at non-toxic doses will produce anti-androgenic effects in rodent male offspring (In vivo extrapolation).*

The alternative approach for predicting AGD

In rats, a reduced AGD in males has been shown to be predictive of adverse effects of the male reproductive system including increased incidence of hypospadias, testosterone decrease and altered reproductive organ weight changes (Bowman et al. 2003, Christiansen et al. 2008, McIntyre et al., 2001, Macleod et al. 2010, van den Driesche et al. 2011, Welsh et al. 2008). Being a predictive marker for other adverse effects on male reproductive development, an effect

on AGD is considered as an adverse effect. AGD has also proven to be a good marker for adverse effects on male reproductive health in humans (Eisenberg et al., 2011, 2012; Eisenberg & Lipshultz, 2015; Mendiola et al., 2015; Thankamony et al 2016). Consequently, it is reasonable to develop an approach that predicts effects on primarily AGD and secondarily other markers of antiandrogenic action using non-animal testing methods, such as in vitro assays and PBK modelling. If not entirely predictive of in vivo effects, such approaches would at the very least be helpful in identifying those anti-androgenic pesticides whose propensity to attain in vitro active concentrations in the fetus would be too low to produce in vivo effects and prioritize the other compounds for testing. If successful, such an approach might help to remove the bottleneck of chemicals that need to be tested and risk assessed for endocrine disruption.

Of the nine pesticides tested in vitro, five showed a relatively marked reduction in testosterone production, in addition to their previously reported AR antagonistic activity. PBK modelling indicated that the internal concentrations at non-toxic maternal doses were in the range of in vitro activity for suppression of testosterone synthesis and AR antagonism. On the basis of these observations, it was reasonable to expect that these pesticides should produce reductions in AGD in the male offspring.

In the in vivo study, all three tested pesticides (fludioxonil, dimethomorph and cyprodinil) showed statistically significant reductions in AGD: fludioxonil and cyprodinil at the middle dose, whereas dimethomorph reduced AGD at the two lowest doses.
The AGD responses agreed with our expectations based on the integrated in vitro / PBK modelling approach, where we anticipated AGD changes at the middle doses. However, none of the three pesticides showed significantly reduced male AGD at the highest dose.

We have demonstrated by a first proof-of-principle study using primarily AGD as an endpoint, that it may be possible to prioritize pesticides for in vivo testing by pinpointing those which are too low in potency and insufficiently prone to reach active concentrations in the fetus. At the same time, we are now in a position to highlight those factors that might complicate straightforward predictions of in vivo activity on the basis of PBK modelling combined with in vitro assays:

In vivo AGD and NR effects
When male rodents are exposed to chemicals with antiandrogenic activity during development, NR is often also affected like AGD in a dose-related manner, and for some potent drugs or pesticides, perinatal exposure can even lead to males having female-like NR (Ostby et al. 1999, Parks et al. 2000, Hass et al. 2007, Christiansen et al. 2008, Christiansen et al., 2009). This is the background of including both AGD and NR as sensitive markers of anti-androgenic action in the OECD test guideline for the extended 1 generation studies (TG 443) and the recently developed reproductive toxicity studies (TG 421/422). In general we did not observe significant effects on NR for any of the pesticides, although a few dosed animals appeared with nipples (but none of the controls). A larger statistical study power with a higher number of animals may have resulted in significant effects. Although the effects on AGD did not appear to increase with dose, we cannot exclude the possibility that higher doses than those applied might affect other androgen-sensitive endpoints such as NR.

For the three pesticides, we observed shallow dose-response patterns on AGD reductions. Other pesticides with anti-androgenic action such as vinclozolin and procymidone have shown steeper and more pronounced dose-response effects on AGD and effects on NR as well as reproductive organ weights (Hass et al., 2007). However, some other chemicals have exhibited rather shallow dose-response curves for AGD or even biphasic dose-response patterns, and the concordance with NR was sometimes lacking. Examples include paracetamol (shallow dose response), finasteride (shallow dose-response), bisphenol A (shallow dose response with only slight effect on NR at the top dose), and butylparaben (shallow dose response, no effect on NR) (Kristensen et al. 2011; Bow-

man et al. 2003; Christiansen et al. 2009, Christiansen et al., 2014; Boberg et al., 2016). Butylparaben is an example of a chemical that affects AGD but not NR similar to the pesticides in this study (Boberg et al 2016). These findings might suggest that chemicals with a mixed target profile may induce only subtle effects on AGD.

Some chemicals like bisphenol (Christiansen et al., 2014) and butylparaben (Boberg et al., 2016) have previously been shown to affect AGD in both males and females. This was also observed for fludioxonil, cyprodinil as well as for dimethomorph in this study. The implications of decreased AGD in female rats is still not fully explored or understood, but it is probably also a sign of endocrine disruption during the critical periods of sexual development. Whether the reduced AGD in females is due to an estrogenic mode of action remains to be determined.

The reason for the relatively weak effects on male AGD and the absence of significant effects at highest doses could be due to:

1) Limited **group size** (n=6-10) and therefore only a low statistical power to identify small responses.
2) Challenges in performing **in vitro-in vivo correlations**
3) **Unusual kinetic profiles** of the pesticides in the rat that may have escaped our modelling efforts
4) **Multiple mechanism of actions** of the chemicals

Ad 1) This is a likely explanation. A more powerful study with more animals per group could clarify possible overlooked effects at high doses. In our earlier studies on potent anti-androgens such as vinclozolin or procymidone, the group size used has been sufficient for demonstrating dose-related effects on AGD and other male reproductive health parameters (Hass et al 2007, Metzdorff et al 2007, Christiansen et al 2008). As the control values in this study are in line with historical control values, unusual control values are therefore not a likely explanation.

Ad 2) We used the IC_{20} for AR antagonism and the LOEC for testosterone inhibition as measures for in vitro activities. These effect levels may have been too low and for future studies we recommend the use of higher effect levels (e.g. IC_{50} values).

All three pesticides had presumably reached IC_{50} for either AR antagonism or testosterone inhibition at the doses where AGD effects were found. However, our in vitro-in vivo correlations assumed a similar level of free active pesticide in the rat fetus and in vitro, which is probably not the case due to differing protein binding in the two matrices. A correction factor should be applied in future studies, but its magnitude remains to be worked out. This cannot explain the lack of a clear dose-response relationship on AGD, but could contribute to the weak in vivo responses as doses may have been underestimated.

Ad 3) The fetal level of active pesticides may not have increased with increasing dose as expected by the PBK model. For instance, high doses may have caused the induction of liver enzymes leading to more rapid excretion or causing formation of new metabolites. As consequence, these processes might have prevented the occurrence of internal concentrations as simulated by the PBK model. However, we have no data evidence e.g. for an unexpected CYP induction, and it therefore remains speculation so far.

Ad 4) Most chemicals have multiple targets and the most potent targets for fludioxonil, cyprodinil and dimethomorph from the ToxCast programme are shown in Table 8: AR antagonism has been reported for fludioxonil and dimethomorph, whereas cyprodinil was reported as non-active. Cyprodinil was the least potent compound on AR among the three in our test system that seems to be more sensitive than that used by US-EPA. However, according to ToxCast, cypro-

dinil is active on the peripheral benzodiazepine receptor (PBR) that is involved in steroidogenesis and therefore can explain the reduced testosterone response. Fludioxonil is active on several estrogen-related targets and thus seems to have both anti-androgenic and somewhat weaker estrogenic properties. The involvement of several of these mechanisms could explain the observed shallow dose-response pattern for AGD and the lack of NR in males, but details of how multiple mechanisms impact on these endpoints remain to be worked out. Other chemicals with a very mixed target profile such as BPA and butylparaben have previously shown subtle effects on AGD.

Table 8. Overview of ToxCast and CTD data for fludioxonil, cyprodinil & dimethomorph

	ToxCast targets or assay endpoints (targets for which EC_{50} ≤ 10 µM, except where stated)	**Comparative Toxicogenomics Database** CTD (human, rat & mouse)
Fludioxonil CASRN: 131341-86-1	• AR antagonism (8.6µM) • Activation of ER response element, ERα-ERβ dimerization & ERβ-homo-dimerization • Inhibition of ERα-induced transcription • Transactivation of hPXR, NURR1, RAR, RXR • Inhibits CYP1A2 & CYP2E1	• AR • ERα, ERβ • PGR, RXR • Apoptosis/cell cycle regulation • microRNA
Cyprodinil CASRN: 121552-61-2	• Binding to rPBR • Binding muscarinic acetylcholine receptors (M3-M5) & norepinephrine transporter • Inhibits CYP1A2 activity	• AR • Metabolism (AhR, ARNT, CYP1A1)
Dimethomorph CASRN: 110488-70-5	• Binding to AR (1.8 µM) • AR antagonism (24.6µM) • Binding vasopressin receptor A1 • Induces inflammation • Inhibits CYPs (2C19, 3A1, 3A2, 3A5)	• AR • Phosphodiesterase (PDE4A)

Numbers in parentheses indicate the AC50/EC50 estimated from curve fits from the assay in question. AR: androgen receptor; rPBR: rat peripheral benzodiazepine receptor; ER: estrogen receptor, PGR: progesterone receptor; RXR: retinoic acid receptor.

Overall evaluation of endocrine disrupting effects

Dimethomorph did not cause any significant effect on NR or reproductive organ weight changes at PND17. But the fact that the compound was most potent on AR antagonism in vitro and on AGD in vivo is in accordance with information from the DAR where dimethomorph has been reported to cause prostate changes, increased testis weight, and Leydig cell tumors in long term studies. Together with the observation that no indications for an estrogenic mechanism of action of dimethomorph exists, we suggest that dimethomorph can be termed an endocrine disruptor that affects male reproductive health.

Cyprodinil appeared in vitro to be mainly a testosterone synthesis inhibitor (probably mediated by PBR) and in vivo we found no other effects up till PND17 – where the animals were killed – than a reduced AGD in males and females. This relatively limited information indicates that cyprodinil has got weak endocrine disrupting effects, although the exact mechanism remains unknown.

Fludioxonil was the compound we studied in most details. According to the DAR fludioxonil has caused increased testis weight in some studies. In our study it showed both AR antagonism and testo-

sterone inhibition in vitro, and others have reported in vitro estrogenicity of fludioxonil. In addition to a slightly reduced AGD in males and females, fludioxonil showed a statistically non-significant tendency towards reduced prostate weights in the male offspring at PND17, but no other clear morphological signs of an anti-androgenic action such as NR were observed in vivo. At the molecular level a few signs of an anti-androgenic action were found: A tendency towards lowered testosterone plasma levels in male offspring, a reduced *Star* expression in testis of 3 months old males (involved in testosterone synthesis) and affected AR in prostates of 3 months old males. Taken together we conclude that fludioxonil has got weak endocrine disrupting effects in vitro and in vivo.

6.3 In vitro/PBK modelling as a tool to prioritize for in vivo testing for AGD effects

The relatively simple PBK models were generally very good in predicting fetal level of the pesticides. This seems very promising for the future for these models.
Concerning the in vitro activity it seems as if AR antagonism may be more important in inducing AGD reductions than testosterone inhibition, at least for the three pesticides. Dimethomorph was the most potent AR antagonist and also the pesticide most potent on AGD changes. The hypothesis that testosterone inhibition is a 'weaker' mode of action for AGD effects than AR antagonismis supported by previous studies on epoxiconazole and tebuconazole, where both were shown to be relatively potent testosterone synthesis inhibitors, but weak AR antagonists, and where both did not affect AGD (Taxvig et al, 2007). Phthalates is a chemical class exhibiting marked effects on male AGD and their primary mode of action has so far been ascribed to testosterone synthesis inhibition. However, our own unpublished data show that some phthalates are also relative potent AR antagonists, indicating dual mechanisms of action of this chemical class.

We used relatively weak in vitro potencies like IC_{20} for AR antagonism and LOECs for testosterone inhibition as target concentrations in the PBK models to simulate the relevant dose ranges for the in vivo studies (reverse dosimetry). Retrospectively, and in response to the weak in vivo effects observed in our study we should perhaps have selected a higher effect concentration in vitro as reference for the PBK simulations.

Concerning the prostaglandin synthesis inhibition in vitro, we found no indications for a simple correlation between this endpoint and AGD. Fludioxonil is the most potent prostaglandin synthesis inhibitor among the three pesticides, whereas dimethomorph showed no effect on prostaglandin synthesis. This does not exclude, however, that this mechanism may be important for some chemicals but not for others. This needs to be studied in more detail in future studies.

Overall, this screening tool has provided valuable data with regards to identifying potential endocrine disrupting pesticides and may be valuable with regards to prioritizing pesticides for in vivo testing.

6.4 Evaluation of long-term health effects of fludioxonil

Hypothesis 4: *Persistent epigenetic effects in terms of DNA methylation will be induced in adult rat offspring after perinatal exposure to a male developmental toxicant (epigenetics).*

Hypothesis 5: *Perinatal programming by exposure to an anti-androgenic pesticide can induce persistent changes in the prostate (thus predisposing the gland to elevated cancer risk).*

We hypothesized that early-life exposure to anti-androgenic pesticides may cause persistent health effects measurable much later in life, in particular on reproductive tissues such as the testis and prostate. Since we also observed a strong trend towards reduced testosterone levels in adult rats after exposure (50% reduction, albeit not statistically significant), we first performed a targeted gene expression analysis on the testes to get some indication on tissue cellularity or Leydig cell function. In general, we observed little changes in gene transcript levels between the different groups, with some indications of smaller reductions in *Star* mRNA in young adults and Cyp17 in the fully mature rats.

We next focused on the prostates as our previous studies have shown effects on this organ after exposure to mixtures of anti-androgenic pesticides or other industrial chemicals (Isling et al., 2014; Boberg et al., 2015). In those studies at higher doses, aging rats showed a shift from the normal age-related epithelial atrophy towards atypical hyperplasia, and males exposed to anti-androgens perinatally had lower incidences of epithelial atrophy and higher scores for atypical hyperplasia than controls of the same age. Thus, we first performed a targeted 84-gene array with factors known to be affecting oncogenic transformation of prostate tissues. In animals at PD17 and 3-monts of age, it was found that a few genes were dysregulated. A subsequent confirmation in all 3 months old animals, key factors such as *Apc*, *Ar*, *Cav1*, *Egfr* and *Gstp1* were verified to be significantly downregulated in the mid- or high-dosed animals.

Thus, additional histological assessments were carried out on prostate tissues. Overall, we found no morphological changes in ventral or dorsal prostates at 11 months of age related to an increased prostate cancer risk. In young adults, however, a slight, albeit not statistically significant increase in scores for inflammation and atypical hyperplasia was seen in the fludioxonil groups. Since a few control animals were also affected, these data are inconclusive, but as induction of inflammation (a secondary and/or modulating effect) has been seen with the potent anti-androgen vinclozolin, this may be a sign of endocrine disruption and therefore calls for further investigation of persistent adverse changes. In our previous anti-androgenic mixture studies, a more pronounced atypical hyperplasia effect was observed at 18 months of age when compared to 10 months old rats (Isling et al., 2014; Boberg et al., 2015). In the current study, the aging rats were 11 months of age, and it is possible that histological investigations at an even later age would have provided different results.

Possibly reflecting the slight effects observed at the histological level at 3 months of age, we observed a significant decrease in a few androgen-sensitive genes in the prostates at that age. Thus, we speculated if early-life exposure to fludioxonil could persistently affect the methylation status of gene promoters. When analyzing known methylation-sensitive sites in the promoter region of *Apc*, *Ar*, *Cav1* and *Gstp1,* however, we did not observe increased methylation, which could explain a downregulation of mRNA levels. In fact, the promoters were frequently methylated also in control animals. Therefore, we have not found any evidence of hypermethylation being the cause of lower transcript abundance. It is important to stress though that this does not exclude that an epigenetic mechanism may be involved in the prostate findings as this study was rather restricted due to resource constraints.

In sum, our data suggest some subtle long-term effects on androgen-sensitive tissues following early-life exposure to fludioxonil, but the data remains inconclusive.

6.5 Comparison to human exposure levels

In this project we aimed at elucidating hazards of the pesticides and to develop an alternative tool for hazard ranking of the pesticides. However, it should be noted that chronic exposures to these pesticides individually are low for the normal consumer. For fludioxonil and dimethomorph, the cumulative dietary exposure of a Danish adult consumer is 0.025 µg /kg bw/day and 0.014 µg/kg bw/day, respectively (DTU report “Pesticide residues, results for the period 2004-2011”). These values are far below the doses used in the in vivo study. However, occupational exposures may result into much higher exposures.

While human exposure to a single pesticide can be considered as low, humans are exposed to more than one pesticide via various intake routes. As different chemicals can exhibit the same effect endpoint, humans are exposed to more than one chemical with an antiandrogenic mode of action, and not only to pesticides, but also to many more every-day chemicals like phthalates, bisphenol A and triclosan. The key question here is whether this load of antiandrogenic chemicals exhibits a combination effect that can affect human health.

7. Conclusions

The main findings were:

- Eight out of nine pesticides known to antagonize the AR, did also affect hormone synthesis in vitro, including testosterone inhibition for seven of the pesticides.
- The PBK modelling approach was very successful in predicting in vivo levels of pesticides in rat fetuses (within a factor of 0.5-1.1)
- The integrated in vitro/PBK modelling approach together with information from risk assessment reports for the pesticides was valuable for ranking and selecting pesticides for in vivo testing
- Analytical chemistry was successful in measuring parent pesticides and metabolites in dams, fetuses and amniotic fluid. Metabolite profiles seemed relatively similar in dams, female and male pups.
- The three pesticides, fludioxonil, cyprodinil and dimethormorph, were predicted to affect AGD in vivo, and verified to cause a significant reduction of AGD in male rats (although without a clear dose-response relationship). The presence of a plausible mode of action underlying the AGD effects together with previous reports on male reproductive organ weight changes (DARs) suggest that the observed AGD reduction is most likely not a random-finding, but true.
- No other morphological or histological signs of anti-androgenic actions were statistically significant
- At the molecular level, fludioxonil was found to affect gene expression in testis (*Star*) and prostates (e.g. *Ar*) and to cause a non-significant 50% reduction in testosterone.
- No identified risk of prostate cancer of fludioxonil was evident by histological analysis, but signs of endocrine disruption in prostates of young rats call for further investigation of persistent adverse changes.
- No identified epigenetic mechanism in prostates was observed. The rather restricted analysis does not exclude though that epigenetic mechanisms may be involved.

In conclusion, we have developed a first proof-of-principle that it may be possible to predict effects on AGD by a combined in vitro and PBK modelling approach. Such a concept may in the future be used for prioritizing the untested pesticides (and non-pesticidal chemicals) for in vivo testing.

Furthermore we have identified three pesticides, fludioxonil, dimethomorph and cyprodinil, with multiple targets in vitro and weak endocrine disrupting effects in vivo.

8. Perspectives

The outcome of this project points to some relevant topics to address in future projects:

- The integrated in vitro/PBK modelling concept seems very promising and needs to be pursued and refined in future projects. The perspective is that this approach can be used for prioritizing the many chemicals for which we still need information on endocrine disruption for in vivo testing.
  In the Table below we have compared the current ADI for the three pesticides with a Point of Departure (POD) divided by an Uncertainty Factor (UF) calculated from the data obtained in this project. This comparison is rough and very early and has to be considered with caution, as the evaluation should include much other information regarding other hazards/effects and dose-response relationships. However the preliminary and estimated POD/UF may give some indications on potential consequences of using an alternative risk assessment procedure. The POD/UF based on AGD reductions in male rat offspring were found to be 2, 0.2 and 1.4 fold lower for fludioxonil, cyprodinil and dimethomorph, respectively. This suggests that the ADIs for fludioxonil and dimethomorph should be reduced based on the current findings of endocrine disruption for these compounds. However, for cyprodinil we have produced no data evidence that would suggest a refinement of the current ADI. For a better comparison, the preliminary refined ADIs are shown below: they are around 2-3-fold lower than the current ADIs for fludioxonil and dimethomorph. These preliminary values should be considered with great caution as these calculations need to be improved and refined in future studies.

	Fludioxonil	Cyprodinil	Dimethomorph
ADI current	0.37 mg/kg (liver & kidney toxicity)	0.03 mg/kg (liver & BW toxicity)	0.05 mg/kg (liver toxicity)
Point of Departure for AGD reduction (UF: Uncertainty Factor) (NOAEL or LOAEL /UF)	0.2 mg/kg (UF:100)	0.2 mg/kg (UF:100)	0.002 mg/kg (UF:300)
Refined ADI based on an animal-free determination of POD	~0.2 mg/kg	no change	~0.01 mg/kg

[$] NOAEL (or LOAEL) for AGD reduction in male rat offspring divided by an uncertainty factor

- The current paradigm states that the major mechanism of action in AGD development is mediated via the AR. However, there are indications from this and other studies that other mechanisms of action may be involved in AGD development (e.g. paracetamol) and studies to address this are needed. Furthermore, the mode of action underlying the reduced AGD in females remains to be determined.

- We observed significant effects on AGD but not on NR in the rat study. An investigation into 21 previous studies with substances of proven anti-androgenic activity revealed that the sensitivity between NR and AGD was in 13 cases comparable and nipple retention the more sensitive in 6 studies (OECD 2015). Only in a few studies AGD was more sensitive than NR, including the studies on BPA and butylparaben. Our current knowledge on chemicals with multiple mechanisms of action indicates that the sensitivity of these endpoints may be influenced by other, yet unexplored factors. It would be relevant to investigate sensitivity differences of various anti-androgenic chemicals on AGD and NR in future studies.

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Appendix 1. Ranking of pesticides based on in vitro investigations

The pesticides were investigated for antagonism of the human androgen receptor in an assay based on MDA-kb2 cells, where IC_{20} for androgen receptor antagonism was determined ($IC_{20\text{-}AA}$).

For assessing effect on testosterone production the pesticides were tested in the H295R steroidogenesis assay. The assay is based on human adrenocortical carcinoma cells that express all the enzymes necessary for steroid production, and which are capable of synthesizing relevant steroids and steroidal sex hormones. Effects on progesterone, 17α-hydroxy-progesterone, androstenedione, testosterone, dehydroepiandrostenedione, estradiol, estrone, cortisol and corticosterone synthesis were analyzed and a Lowest observed effect concentration (LOEC) for testosterone inhibition was determined ($LOEC_{testo}$). This hormone is considered the most important hormone when it comes to male sexual development during fetal life.

According to our knowledge from previous in vitro-in vivo correlations, pesticides with a potent effect on AR antagonism ($IC_{20\text{-}AA}$ < 1μM) or on testosterone inhibition ($LOEC_{testo}$) are likely to have in vivo adverse effects on male reproductive health. These endpoints were therefore evaluated as being of equal importance when effects were evident at low concentrations. However, an effect on both endpoints was considered of higher priority than an effect on only one of these endpoints.

Thus the priority of the pesticides when taking only in vitro data into account:

Priority 1: $LOEC_{testo}$ <2μM and $IC_{20\text{-}AA}$ <1μM (fludioxonil)

Priority 2: $IC_{20\text{-}AA}$ <1μM (dimethomorph)

Priority 3/4: $LOEC_{testo}$ <2μM and $IC_{20\text{-}AA}$ 1-5μM (quinoxyphen, imazalil)

Priority 5: $LOEC_{testo}$ 2-15μM and $IC_{20\text{-}AA}$ 1-5μM (fenhexamide)

Priority 6: $LOEC_{testo}$ <2μM and $IC_{20\text{-}AA}$ 5-15μM (cyprodinil)

Appendix 2. PBK modelling

PBK Model

The main model aim was to determine by mathematical simulation the most likely intake dose (or dose range) that is expected to result into in vitro active fetal concentration levels (reverse dosimetry). Therefore we developed a simple physiologically based toxicokinetic (PBK) model which simulates the age-dependent physiological and biochemical changes in rodents associated with ongoing pregnancy after repeated daily oral dosing of environmental compounds. Only those maternal tissues and kinetic processes were included which were considered as essential for the estimation of exposure levels in the fetus. The PBK model was implemented in Berkeley Madonna, with no variability terms added to the model. Thus, all simulations represent only average plasma exposure levels. The resulting systems of mass-balance differential equations were solved by numerical integration using the Rosenbrock Algorithm for stiff systems.

Model structure

A generic PBK model consisting of compartments representing the most important maternal tissues (plasma, gut, kidney, liver, fat, plus two lumped compartments) was adopted from O'Flaherty et al. (1992) with several physiological modifications suggested by Emond et al. (2004). The compartments were selected for their pharmacokinetic/dynamic relevance, with liver and kidney as the major sites of elimination and metabolism, fat tissue to account for a potential lipophilicity, blood/plasma for the description of the systemic circulation, and two remaining compartments which include all other well- or poorly-perfused organs and tissues lumped together, for the calculation of the mass balance. This minimum set of compartments was considered as flexible enough to fit the model structure to published data in order to estimate all relevant model parameters.

The PBK model involves a model extension that describes the transplacental transfer of chemicals to the fetus during gestation, which is utilized by the addition of two extra mechanistic placental units, yolk sac and chorioallantoic placenta, and a fetal compartment, which all run in parallel to the other maternal compartments (Figure 2). Here the whole fetus is modeled rather than individual tissues, mainly due to lack of available tissue volume and chemical distribution data as well as an uncertainty in how to describe the dynamic tissue differentiation over short time. Therefore we treated the whole fetus as a single diffusion-limited compartment.

We considered the rate of transport of chemical into the maternal tissue limited by blood flow to that particular tissue, and adapted therefore a flow-limited model structure which is typically preferred for nonvolatile chemicals. As all pesticides are nonvolatile, the concentration of chemical in venous blood was assumed equal to the concentration of chemical in arterial blood; therefore, a lung compartment was not included.

Model-predicted growth of placenta and maternal body weight were modeled according to the equations of O'Flaherty et al. (1992), with differences in gestation time, pup birth weight, and litter size incorporated in the model. As the maternal body weight increases significantly during the relatively short gestation time in the rat (21 days), with the majority of weight resulting from increasing volumes of the placenta, mammary gland, fat, and total fetal volume, this model describes the total change in maternal body weight as the sum of the changes in these four tissue volumes and the initial (non-pregnancy) body weight. Placental volume is described as a sum of three stages of growth, involving changes in both the yolk sac and chorioallantoic placenta. Temporal changes in maternal cardiac output during gestation are modeled as the sum of initial cardiac output and the change in blood flow to the placenta, mammary and fat tissues. Changes in the fractional cardiac output to the mammary gland, fat and yolk sac were assumed to be proportional to changes in tissue volumes, with the exception of the chorioallantoic placenta which increased more rapidly than the tissue volume. During gestation, exposures are allowed to move freely between the maternal and placental plasma. The blood exchange between the placenta and the fetus is activated on GD6.

As the most critical parameter in terms of model uncertainty we identified the transfer between both placenta structures and the fetus. Here we followed the common assumption of a passive diffusion and used first-order clearance rates to describe the bi-directional placental transfer process (from mother to fetus and fetus to mother). Due to missing data we did not consider any elimination processes in the fetus, and therefore interchanges between fetal levels and amniotic fluid were excluded from the model structure.
For all pesticides, active uptake into the gastrointestinal (GI) tract and the absorption from the GI tract into the liver was described by first order kinetics, assuming a 100% oral absorption of the intake dose. The transfer inclusion into the internal blood flow was controlled by a first-order fecal excretion rate. Biliary excretion of the parental compound into the duodenum is modeled as a simple clearance rate from the liver to the intestine (enterohepatic recirculation) with no time delay. Transport of the compound into the tissues from the plasma was modeled using diffusion-limitation via partition coefficients and blood flows. Systemic clearance was considered to occur only in liver and kidney, and represented by first order clearance rates.
Enterohepatic circulation was considered as viable possibility because some pesticides are conjugated with molecular weight above 325, which is often considered as lower bound for molecular weight for enterohepatic circulation in the rat (Guthrie and Hodgson, 1987).
Once ADME parameters were evaluated for the non-pregnant female rat, they were fixed and only parameters related to physiology (e.g. body weight, blood flow, GFR etc.) were modified in order to mimic the physiology of pregnant dams.

Parameterization

Physiological parameters

Most model parameters for physiological components such as tissue volumes, cardiac output, and blood flows were set based on data presented by Brown et al. (1997) and O'Flaherty et al. (1992). Whenever possible, they were updated with data derived from our previous pharmacokinetic studies. The body weight of a non-pregnant rat at GD0 was set as 197 g, and the mean fetal body weight in dependence of the gestational day (GD) was described as Weight(fetus) [kg] = 0.000005092*EXP(0.6413*GD)/1000. The number of fetuses per damn was set to 10. The same physiological model setup was used for all compounds.

Biochemical parameters

Almost all compound specific ADME parameters were extracted and estimated from literature, and if possible we prioritized kinetic information provided by the DAR reports as main source. As these data are usually measured in non-pregnant female adults, we used our model structure at stage GD0 to fit the relevant kinetic parameters to the reported measurements. Parameters values were estimated by adjusting the parameters to obtain the best visual fit of the model to reported time course data. At this stage of model development and evaluation a statistical program was not used to estimate model parameters, i.e. fitting of parameters refers to the manual process of determining one set of parameters that could consistently recapitulate a large base of diverse data published in the DAR reports. This approach of model parameterization by visual inspection cannot guarantee the estimation of an unique set of best-fit parameters, and therefore alternative sets of parameters might exist that can describe the reported time course data similarly well. For example, for the portal GI absorption rate into liver we started the "fitting" process always with a default value of 1 L/hr, and optimized the remaining parameters to achieve the best agreement with the reported measurements. For none of the pesticides we found profound reasons to change this absorption rate constant, and thus decided to accept this value as best possible solution for all pesticides (see Table 1 for final values for the kinetic parameters). It should be noted that the available data differed substantially between the pesticides with respect to their reported details, data amounts and number of independent repeated studies, with sometimes no data available within the first 12 hours after the

animal dosing. As consequence we can expect in these cases an even larger number of different parameter sets with similar good agreements. A possible AR antagonism mediated by the metabolites of the selected pesticides was judged as unlikely, and consequently the target fetal concentration refers always only to the parental compound.

Physicochemical parameters

The tissue:plasma partition coefficients of the pesticides in equilibrium were derived from tissue-composition based equations according to Poulin and Theil (2002). They are based on compound-specific input parameters (vegetable oil-water partition coefficients for adipose tissue, octanol-water partition coefficient for all other tissue compartments, unbound fraction in plasma) and tissue input parameters (volume fractions of water, neutral lipids and phospholipids), and main assumptions are a homogenous drug distribution, with tissues considered as a mixture of total lipids, and water and proteins at global pH 7.4. The slowly perfused tissues:plasma partition coefficient was set equal to the predicted muscle: plasma partition coefficient, and the rapidly perfused tissues:plasma partition coefficient was set equal to the predicted heart:plasma partition coefficient. For the placenta transfer we used always the rapidly perfused tissues:plasma partition coefficient. All information was extracted from literature. All parameters were assumed to be unchanged during pregnancy.

Model prediction of fetal exposures

The time from GD15 to GD18 was defined as most critical during the male programming window, and fetal concentrations were estimated over this period only. Here they were quantified as Area Under Curve (AUC) per hour, as this estimation of an average concentration in the fetal compartment agreed well for AR antagonist such as vinclozoline and prochloraz when we compared their reported in vitro AR activity concentration with model simulation derived from intake doses that have been demonstrated to produce effects on landmarks of male sexual differentiation (e.g. changes in AGD and retained nipples).

To our knowledge no predictive *in silico* method exists which would allow the estimation of the compound-specific transplacental rates for our test species and selected model structure, and as compromise we used the lowest and highest values from literature which have been reported for similar study designs, and defined them as pragmatic “worst-case” space for this clearance rate parameter (0.005 - 2 L/hr). In addition, differences in the rates between the transplacental transfer from the placenta to the fetus and from the fetus to the placenta have been reported, and, if true but ignored, are likely to cause hugely biased estimated fetal levels. Therefore we set a window of equally possible parameters, with maximal relative differences between both clearance rates of ±20%. Consequently, we always simulated at a given intake dose a range of fetal concentrations, with each individual value in this range having the same weight: a minimum estimate refers usually to the combination of a clearance rate of 0.005 l/hr to the fetus and 0.006 l/hr back to the placenta, and a maximum estimate to 2 l/hr to the fetus and 1.6 l/hr back to the placenta.

Table A1: Compound-specific kinetic model parameters

Compound	Portal GI absorption rate into liver (L/hr)	biliary excretion rate (L/hr)	Fecal excretion rate from gut (L/hr)	Urine excretion rate from kidney (L/hr)	Rate of metabolism in liver (L/hr)
Dimethomorph	1	-	2	0.01	0.3
Fenhexamid	1	-	3	0.05	0.15
Quinoxyfen	1	10	2	0.01	0.3
Cyprodinil	1	10	1	0.2	0.01
λ-Cyhalothrin	1	-	4	0.05	0.3
Pyrimethanil	1	-	0.1	0.01	0.8
Fludioxonil	1	-	3	0.01	0.3
Imazalil	1	-	0.5	0.1	0.3
o-Phenylphenol	1	-	0.1	0.01	0.2

Table A2: Tissue:plasma partition coefficients

Compound	Adipose	Kidney	Liver	well-perfused	poorly-perfused
Dimethomorph	3.47	3.34	3.62	2.85	2.12
Fenhexamid	7.60	5.52	6.06	4.66	3.40
Quinoxyfen	2.30	6.02	6.62	5.10	3.70
Cyprodinil	1.60	5.70	6.30	4.80	3.50
l-Cyhalothrin	24.80	6.40	7.00	5.40	3.90
Pyrimethanil	1.05	3.60	3.90	3.10	2.30
Fludioxonil	1.90	5.90	6.40	4.90	3.60
Imazalil	11.07	5.70	6.26	4.80	3.50
o-Phenylphenol	3.72	4.47	4.89	3.80	2.77

Appendix 3. LC-QTOF quantification of parent pesticides and metabolites

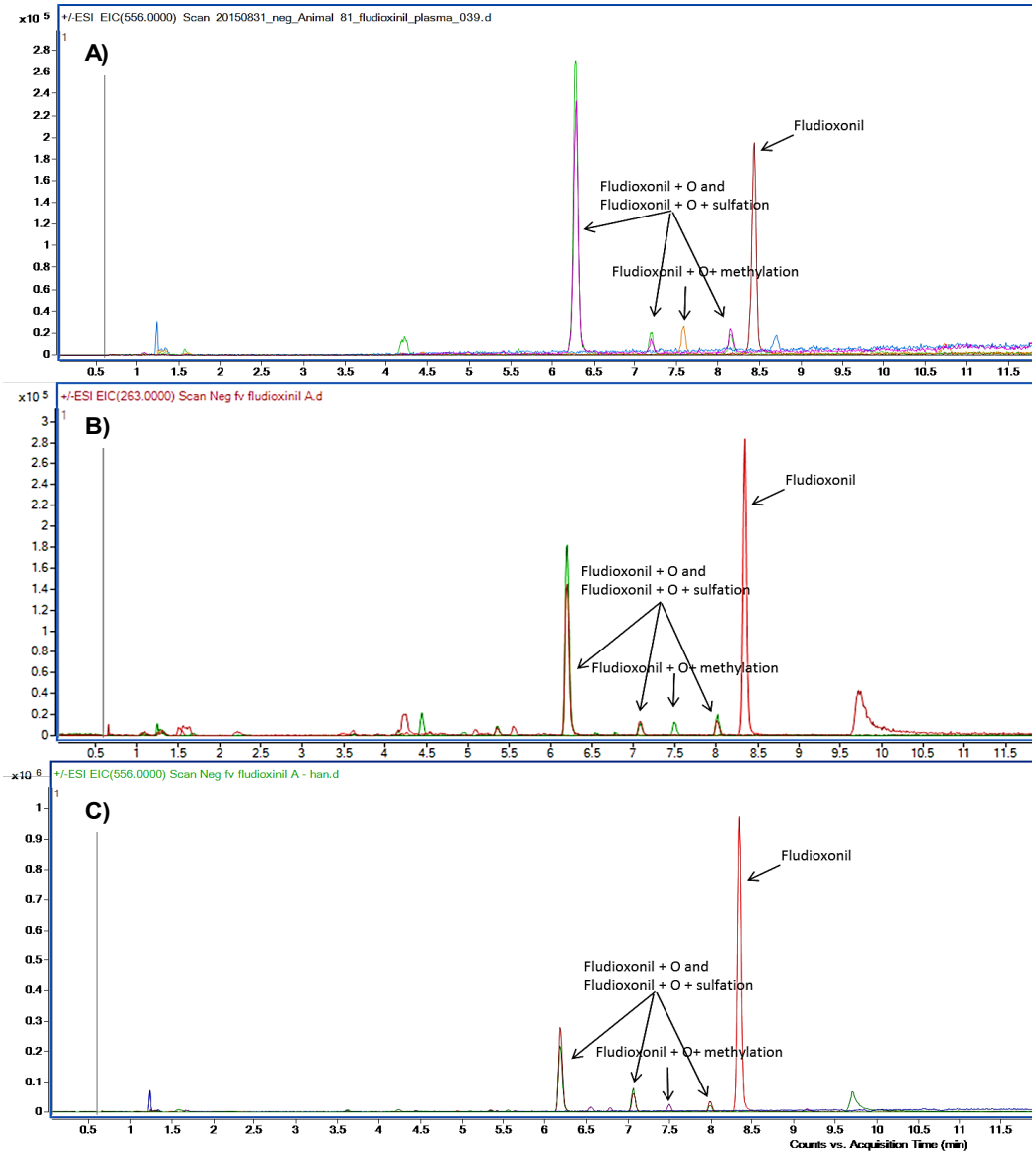


Fig. 3.1: Chromatographic data showing levels of fludioxonil and its metabolites at GD21 after exposure to 60 mg/kg fludioxonil from GD7 to GD21. A: plasma level in a dam, B: the amniotic fluid, and C: plasma level in a male fetus (N=1).

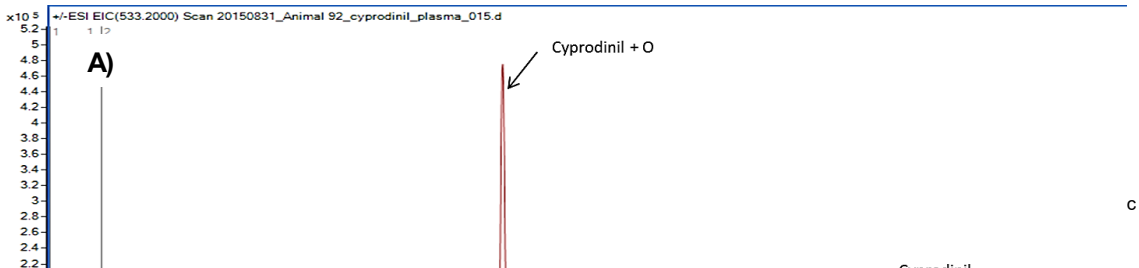

+/-ESI EIC(533.2000) Scan 20150831_Animal 92_cyprodinil_plasma_015.d
A)
Cyprodinil + O

Fig. 3.2: Chromatographic data showing levels of cyprodinil and its metabolites at GD21 after exposure to 60 mg/kg cyprodinil from GD7 to GD21. A: plasma level in a dam, B: the amniotic fluid, and C: plasma level in a male fetus (N=1).

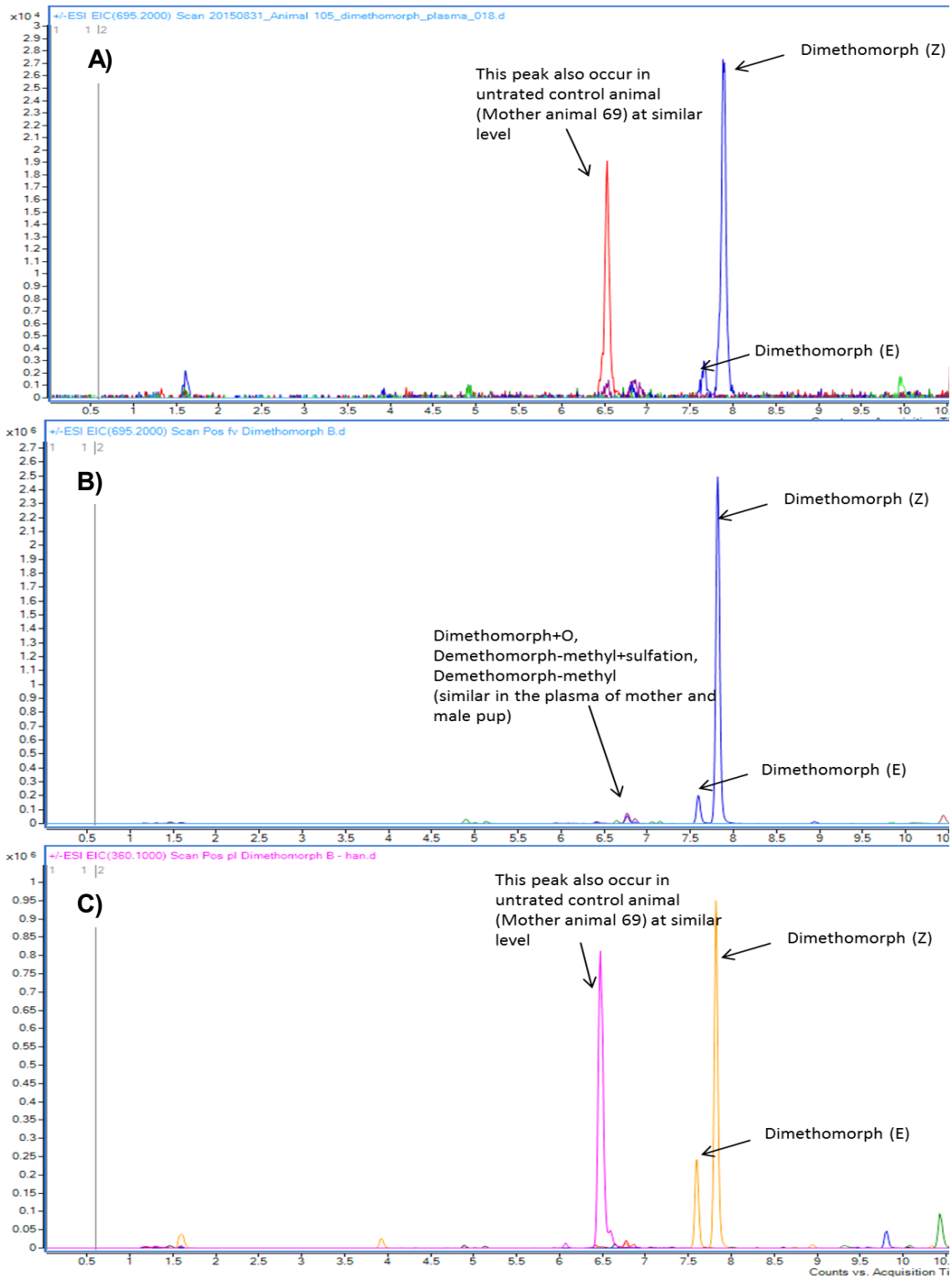

+/-ESI EIC(695.2000) Scan 20150831_Animal 105_dimethomorph_plasma_018.d
A)
This peak also occur in untrated control animal (Mother animal 69) at similar level
Dimethomorph (Z)
Dimethomorph (E)
+/-ESI EIC(695.2000) Scan Pos fv Dimethomorph B.d
B)
Dimethomorph (Z)
Dimethomorph+O, Demethomorph-methyl+sulfation, Demethomorph-methyl (similar in the plasma of mother and male pup)
Dimethomorph (E)
+/-ESI EIC(360.1000) Scan Pos pl Dimethomorph B - han.d
C)
This peak also occur in untrated control animal (Mother animal 69) at similar level
Dimethomorph (Z)
Dimethomorph (E)
Counts vs. Acquisition Ti

Fig. 3.3: : Chromatographic data showing levels of dimethomorph and its metabolites at GD21 after exposure to 20 mg/kg dimethomorph from GD7 to GD21. A: plasma level in a dam, B: the amniotic fluid, and C: plasma level in a male fetus (N=1).

Appendix 4. In vitro data

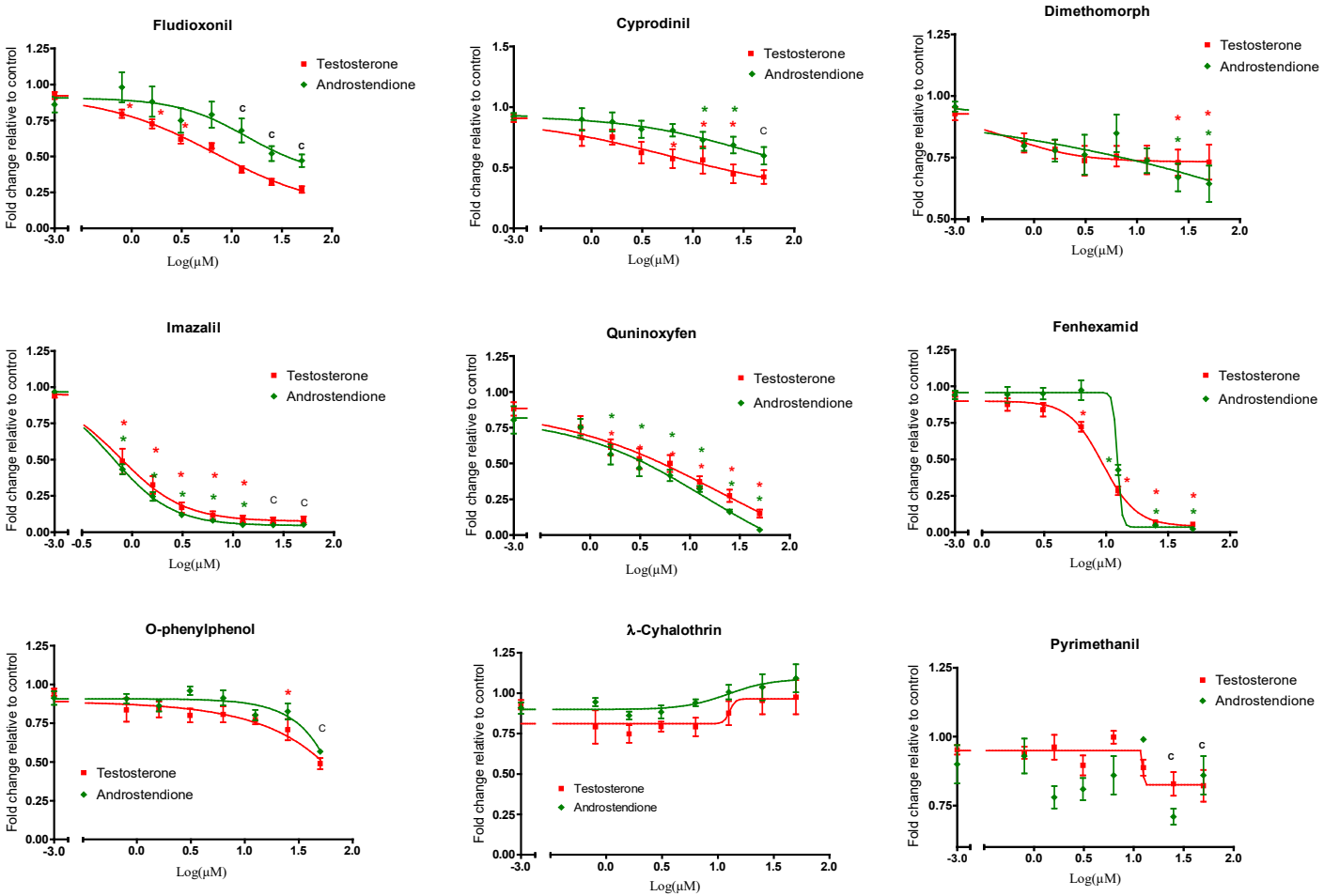


Fig. 4.1. Results from the H295R cell assay showing the effects of the the nine pesticides on the production of testosterone (red) and androstendione (green). Data represents Mean ± SEM of normalized data. * indicated statically significantly different compared to background control. C indicates measured cytotoxicity.

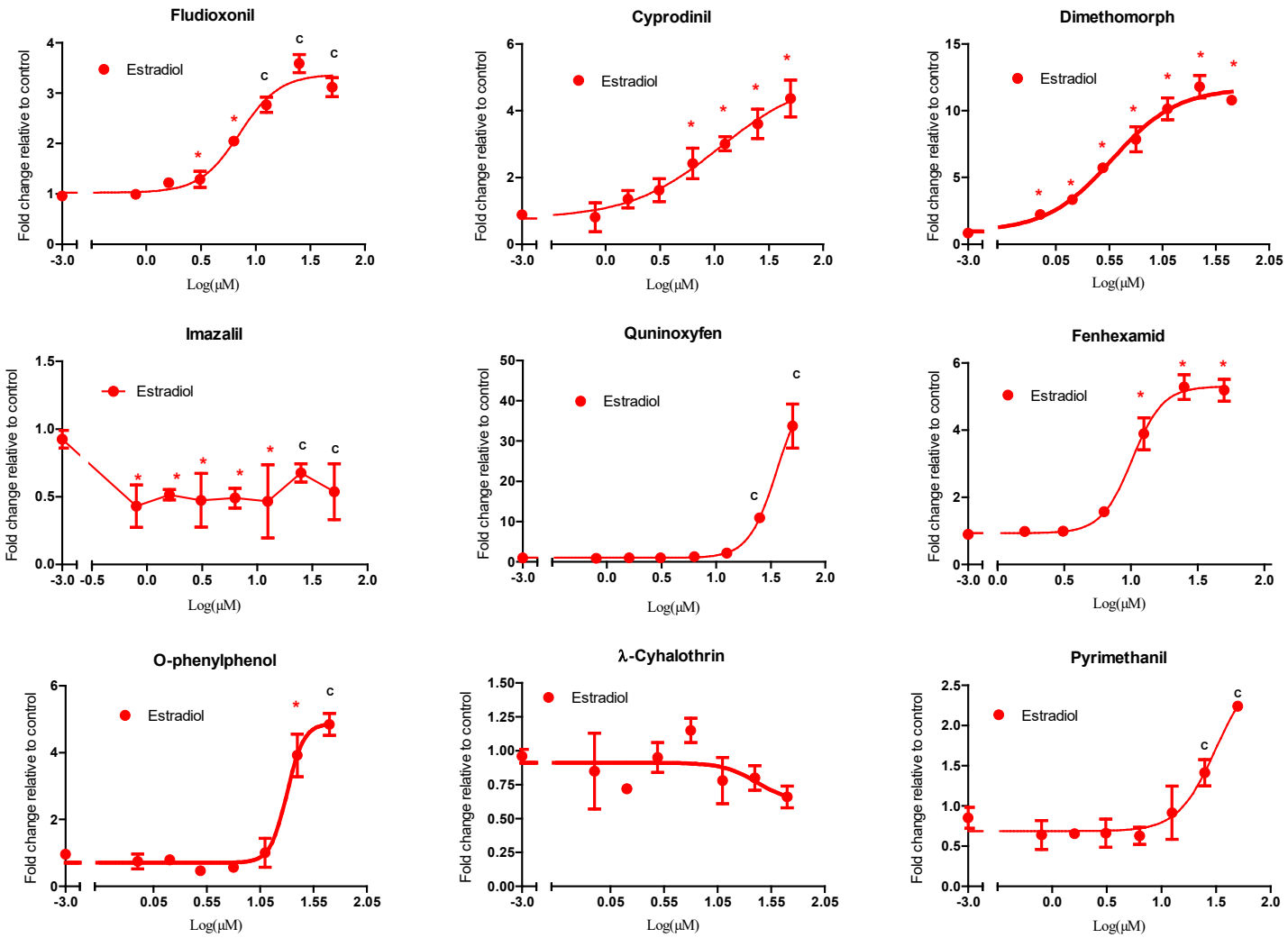


Fig 4.2: Results from the H295R cell assay showing the effect of the nine pesticides on estradiol production. Data represent mean± sd of normalized data.
* indicate statistically compared to background control. C indicates measured cytotoxicity.

Appendix 5. In vivo data

App 5.1 In vivo organ weights

Table A3. Male reproductive organ weights at 17 days of age after perinatal exposure to Fludioxonil (Flu), Cyprodinil (CY) or dimethomorph (DIM). No statistically significant differences between controls and exposed groups were observed in a Dunnett's test for each compound compared to controls (adjusted for body weight variations as a covariate). LABC: levator ani/bulbocavernosus muscle. Means ± SD are shown.

	N	Body weight (g)	Pooled testes (mg)	Epididymis (mg)	Prostate (mg)	Seminal vesicle (mg)	LABC (mg)	Bulbourethral gland (mg)	Liver (mg)	Adrenals (mg)
Control	8	32 ± 5.5	122.8 ± 19.9	27.1 ± 3.1	16.3 ± 3.7	9.6 ± 8.0	28.2 ± 8.0	1.8 ± 0.4	849 ± 156.6	27.1 ± 3.1
FLU-20	10	34 ± 4.7	125.0 ± 14.3	27.3 ± 5.6	17.0 ± 4.3	11.3 ± 7.9	30.2 ± 7.9	2.0 ± 0.2	900 ± 142.6	27.3 ± 5.6
FLU-60	8	35 ± 5.4	128.0 ± 17.5	26.8 ± 4.8	15.5 ± 2.6	9.2 ± 4.6	28.8 ± 4.6	1.9 ± 0.6	875 ± 150.5	26.8 ± 4.8
FLU-180	6	31 ± 5.7	120.9 ± 23.7	25.8 ± 3.4	14.4 ± 3.1	10.8 ± 3.6	27.9 ± 3.6	1.8 ± 0.5	793 ± 160.1	25.8 ± 3.4
CY-20	9	32 ± 5.5	119.9 ± 17.6	23.4 ± 4.9	15.7 ± 2.6	10.2 ± 8.7	29.4 ± 8.7	1.9 ± 0.5	801 ± 182.9	23.4 ± 4.9
CY-60	9	40 ± 6.7	149.3 ± 20.2	28.2 ± 2.5	18.6 ± 3.3	13.8 ± 6.5	27.7 ± 8.1	2.4 ± 0.5	1075 ± 215.0	28.2 ± 2.5
CY-180	7	32 ± 6.2	127.7 ± 14.9	26.8 ± 2.5	14.5 ± 1.8	8.9 ± 2.0	27.5 ± 5.5	1.8 ± 0.3	867 ± 162.5	26.8 ± 2.5
DIM-6.7	8	33 ± 7.3	124.8 ± 28.7	25.5 ± 4.7	16.2 ± 4.4	9.1 ± 2.2	31.1 ± 6.3	1.9 ± 0.6	860 ± 230.3	25.5 ± 4.7
DIM-20	7	33 ± 3.4	131.2 ± 13.9	28.9 ± 3.0	16.6 ± 3.8	9.2 ± 2.2	29.3 ± 4.2	2.1 ± 0.9	875 ± 97.7	28.9 ± 3.0
DIM-60	9	31 ± 4.0	117.0 ± 12.7	25.4 ± 3.5	14.7 ± 2.9	9.1 ± 2.8	29.7 ± 7.4	1.8 ± 0.3	806 ± 140.5	25.4 ± 3.5

Table A4. Relative male reproductive organ weights 17 days of age after perinatal exposure to Fludioxonil (Flu), Cyprodinil (CY) or dimethomorph (DIM). No statistically significant differences between controls and exposed groups were observed in a Dunnett's test for each compound compared to controls. LABC: levator ani/bulbocavernosus muscle.

	N	Both testes (mg/100 g)	Epididymis (mg/100g)	Prostate (mg/100g)	Seminal vesicle (mg/100g)	LABC (mg/100g)	Bulbourethral gland (mg/100g)	Liver (mg/100g)	Adrenals (mg/100g)
Control	8	387.1 ± 17.0	86.7 ± 14.0	51.6 ± 10.3	29.7 ± 5.4	77.6 ± 33.7	5.7 ± 1.0	2671.2 ± 164.4	29.6 ± 2.8
FLU-20	10	372.0 ± 28.9	73.1 ± 28.2	50.1 ± 9.1	33.7 ± 6.3	79.5 ± 33.9	5.2 ± 2.1	2658.1 ± 105.0	27.9 ± 3.3
FLU-60	8	374.4 ± 24.2	79.6 ± 12.2	45.4 ± 6.7	27.1 ± 4.2	85.1 ± 14.7	4.9 ±2.3	2606.5 ± 194.8	23.2 ± 10.6
FLU-180	6	385.6 ± 32.6	83.0 ± 5.0	45.8 ± 2.5	28.3 ± 15.7	90.5 ± 12.7	5.7 ± 1.4	2519.1 ± 156.6	26.5 ± 3.9
CY-20	9	382.0 ± 30.7	74.6 ± 10.9	50.1 ± 6.6	32.5 ± 6.6	92.2 ± 19.4	6.0 ± 0.9	2532.9 ± 312.9	27.4 ± 10.7
CY-60	9	381.6 ± 50.0	72.5 ± 10.8	47.2 ± 6.5	28.3 ± 25.7	70.2 ± 21.8	6.0 ± 1.3	2695.7 ± 112.1	26.4 ± 2.9
CY-180	7	400.0 ± 34.7	83.9 ± 14.6	45.4 ± 6.2	27.8 ± 5.4	75.2 ± 37.9	4.3 ± 2.4	2691.0 ± 109.9	21.3 ± 5.4
DIM-6.7	8	382.6 ± 23.5	78.8 ± 6.3	49.5 ± 6.3	19.8 ± 12.8	97.8 ± 21.9	5.3 ± 2.8	2619.7 ± 179.2	26.4 ± 6.7
DIM-20	7	393.4 ± 15.4	87.0 ± 9.6	49.9 ± 11.1	27.6 ± 6.2	74.1 ± 34.5	5.2 ± 3.2	2625.0 ± 119.5	28.6 ± 4.5
DIM-60	9	382.7 ± 14.8	83.2 ± 9.7	48.3 ± 9.2	29.4 ± 7.3	97.7 ± 26.8	6.1 ± 1.1	2620.6 ± 149.2	27.3 ± 4.3

App 5.2 Histological assessments of prostates

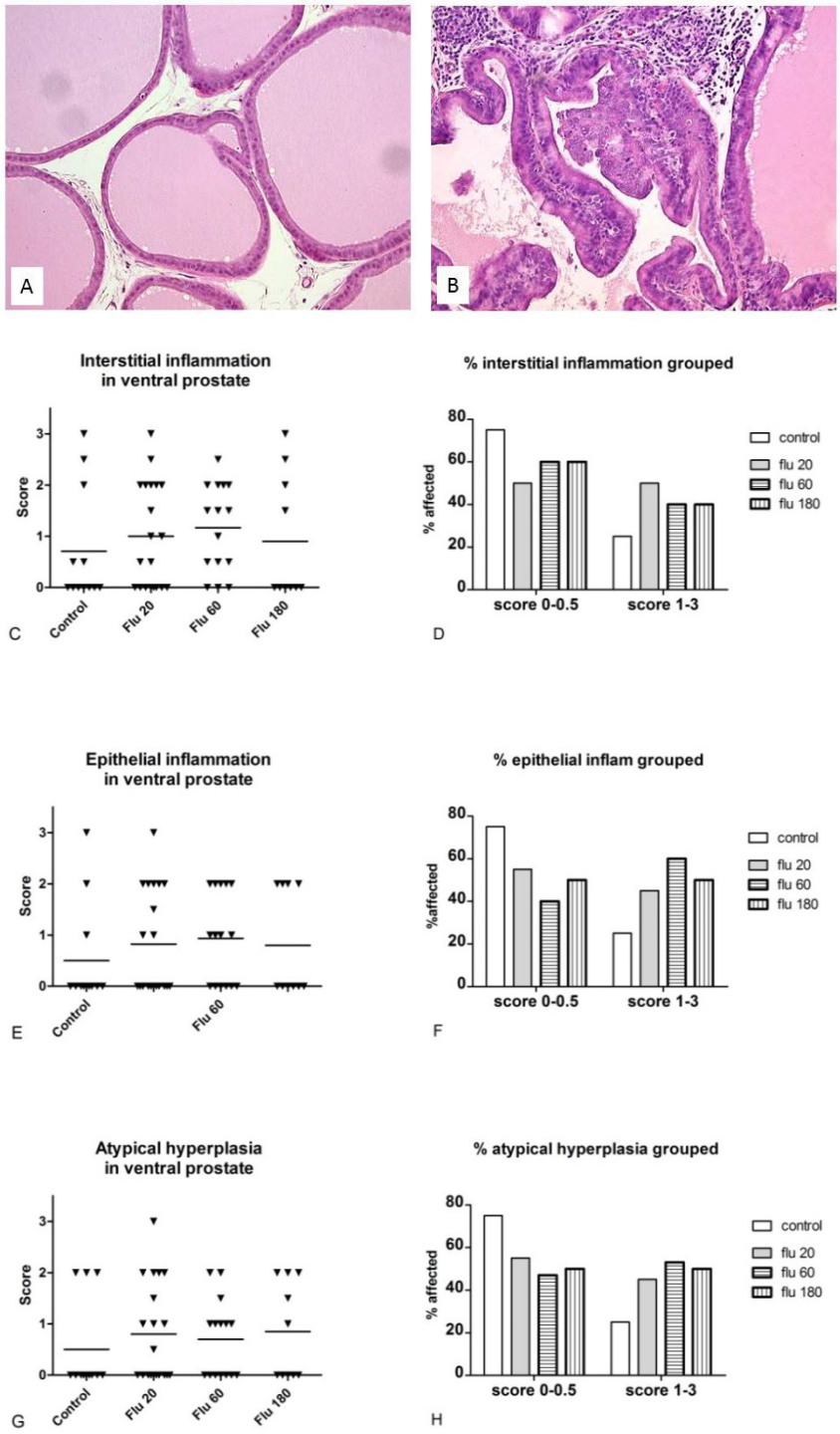
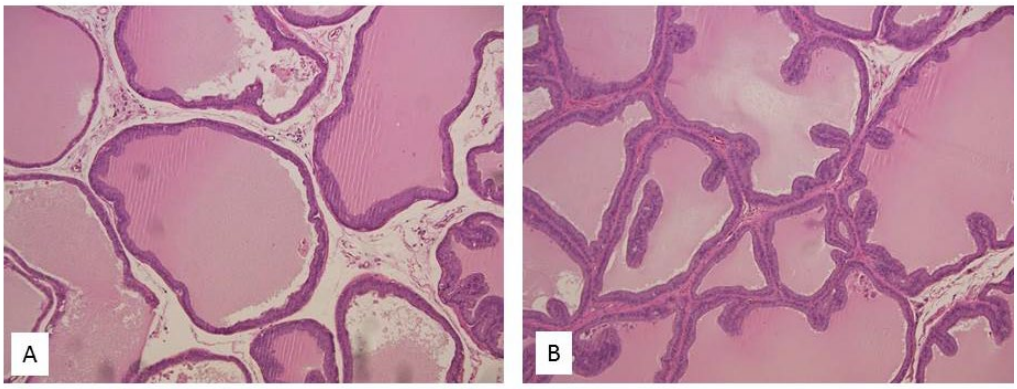


Fig. 5.1 Ventral prostate histology at 3 months of age following perinatal exposure to 0, 20, 60 or 180 mg/kg bw/day of Fludioxonil. In all groups a large variation between individuals was seen with the majority of prostates showing a normal morphology with presence of large, unfolded acini with columnar epithelium in the central part of the tissue and smaller acini with more folded, tall columnar epithelium in the periphery (A). In all groups, a number of animals showed marked interstitial inflammation (arrow) often associated with epithelial invasion and reactive atypical hyperplasia with multiple layers of apolar cells with prominent nucleoli (asterisk) (B). Scores for these findings showed marked changes in 3 of 12 controls while approximately half the Fludioxonil exposed animals were affected (C, E, G). There were no statistically significant differences between groups when comparing individual scores or grouped scores (D, F, H). A and B are from control animals with scores 2 for all findings; original magnification 20x. C-E: plot of scores for each finding

(individual values). F-H: column diagrams showing incidence of findings grouped as low (0-0.5) or high (1 to 3) scores (percentage of animals in each with a given score).

Morphometric analyses were carried out for controls and the middle dose of FLU to evaluate possible differences between groups regarding distribution of compartments (epithelium, lumen or stroma). All measures were comparable between dose groups (data not shown).

At 11 months of age no clear effects of FLU treatment could be observed by histological examination of ventral prostates. Generally, low scores for atypical epithelial hyperplasia was seen compared to the examination at 3 months of age, and no interstitial inflammation was observed. More animals had low scores for epithelial thickness in the middle dose FLU group than in the control group. Additionally, high scores for epithelial atrophy were seen in 4-5 animals in each of the two lowest FLU groups, but not in the control group or the high dose FLU group (Fig. 5.2). These findings were not statistically significant, but could point to more prominent atrophy in these dose groups compared to controls. This was followed up by a morphometrical analysis to investigate possible differences in distribution of compartments, but revealed no statistically significant differences between dose groups (Fig. 5.3). All in all, there were no indications of increased hyperplasia or increased epithelial height, but rather an impression of lower epithelia and more atrophy in the lowest FLU dose groups.


A
B

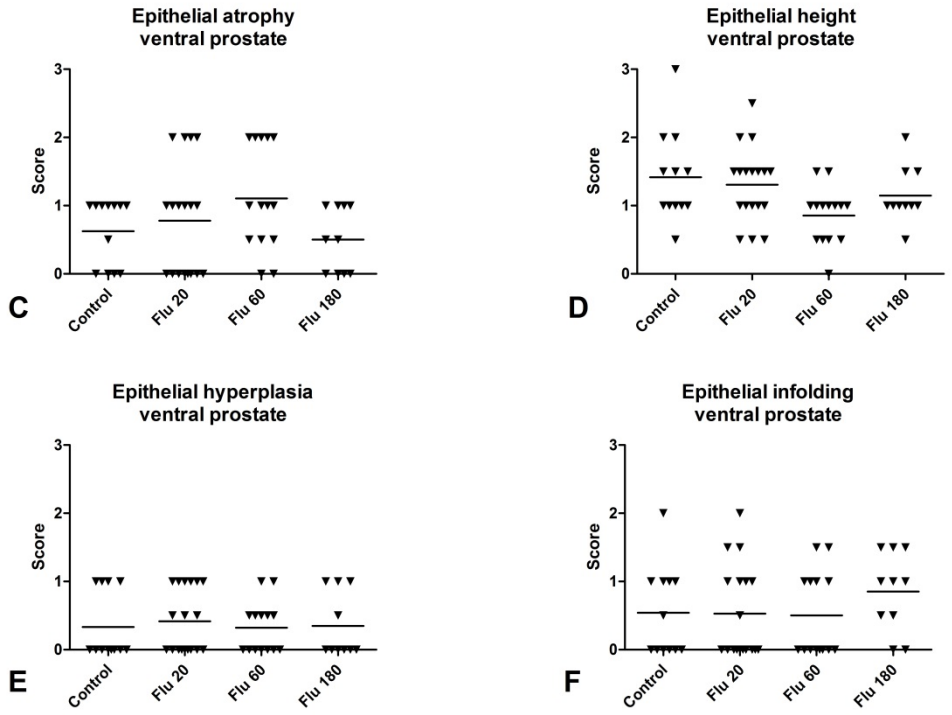


Fig. 5.2. Ventral prostate histology at 11 months of age following perinatal exposure to 0, 20, 60 or 180 mg/kg bw/day of Fludioxonil. In all groups, most animals showed a normal morphology with large acini with varying epithelial height and some degree of epithelial atrophy (A), whereas hyperplastic areas did not show cellular atypia, but a homogeneous epithelium with marked infolding (B). The distribution of scores for these findings was comparable between groups (C-F).

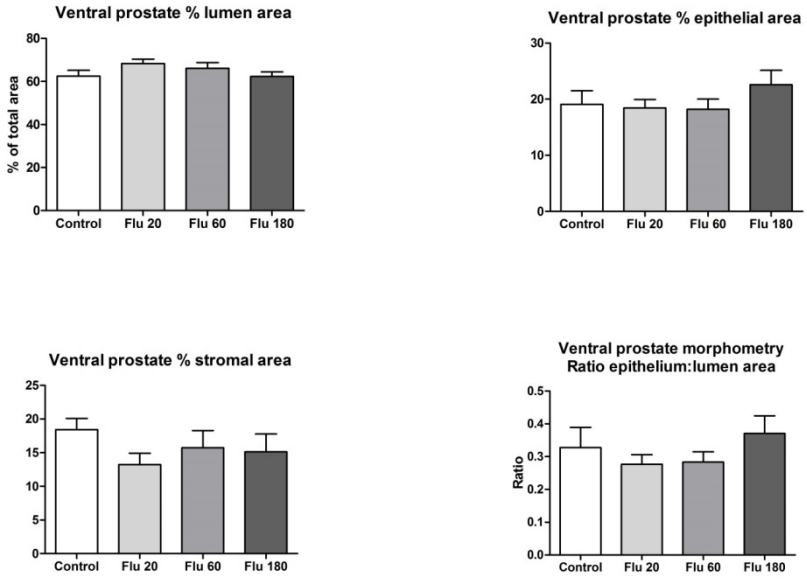


Fig 5.3. Assessment of relative area of different compartments of ventral prostate at 11 months of age. Mean ±SD. No significant differences between groups were observed.

To potentially gain some further knowledge on endocrine effects on the prostate, we also examined the dorsal prostate, which is considered to be particularly sensitive to changes in estrogen levels; as opposed to the ventral prostate which is considered more sensitive to changes in androgen levels (OECD 2009). Interstitial inflammation was not observed in the dorsal, lateral and anterior prostate. This could suggest that estrogen and androgen levels were normal at the time of examination, which was further supported by the lack of changes in male reproductive organ

weights at 3 and 11 months of age. No differences between dose groups were observed regarding epithelial morphology, folding of epithelium or cell sloughing at this age.

Prediction of persistent health effects caused by widely used anti-androgenic pesticides

The majority of the 353 currently used pesticides within the EU have not been tested for sensitive endocrine endpoints such as anogenital distance (AGD). Since it is not feasible to test all of these pesticides by using in vivo methods within the foreseeable future, the development of alternative approaches for predicting endocrine disrupting effects is imperative. In this context, the morphometric measure AGD has proven to be a non-invasive, life-long biomarker for adverse effects on male reproductive health in animals and humans. The aim of this project was to develop and validate a novel approach for predicting effects on AGD based on in vitro profiling in combination with physiologically-based kinetic (PBK) modelling. PBK models were developed for nine pesticides and used to predict the in vivo doses necessary to obtain the expected critical concentrations in the rat fetus. The pesticides fludioxonil, cyprodinil, and dimethomorph, which all are androgen receptor antagonists and inhibitors of testosterone synthesis in vitro, were selected for validation in a rat developmental study. AGD was, as predicted, slightly reduced in male pups and the fetal concentrations of pesticides were shown to be within a factor of 2 from the PBK model prediction levels, demonstrating the feasibility of the approach.
In conclusion, we have identified three pesticides as being weak endocrine disruptors and have demonstrated the utility of the approach by a proof-of-principle study showing positive predictions for male reproductive health effects. This concept may be used in the future for prioritizing the 353 pesticides for further in vivo testing.

Dansk resume:

Hovedparten de 353 anvendte pesticider i EU er ikke blevet undersøgt for følsomme endokrine endpoints så som anogenital afstand (AGD). Da det ikke er muligt at teste alle disse pesticider ved hjælp af in vivo forsøg inden for en overskuelig fremtid, er udvikling af alternative metoder til at forudsige hormonforstyrrende effekter nødvendig. Det morfometriske endpoint, AGD, har vist sig at være en non-invasiv, livslang biomarkør for skader på mandlig reproduktiv sundhed hos dyr og mennesker. Formålet med dette projekt var at udvikle og validere en ny tilgang til at forudsige effekter på AGD baseret på in vitro profilering i kombination med fysiologisk-baseret kinetik (PBK) modellering. PBK modeller blev udviklet for ni pesticider og anvendt til at forudsige de in vivo doser, der er nødvendige for at opnå en forventet kritisk pesticidkoncentration i rottefostret. Pesticiderne: fludioxonil, cyprodinil, og dimethomorph, som alle er androgen receptor antagonister og hæmmer testosteronsyntese in vitro, blev udvalgt til validering i drægtige rotter. AGD var, som forudsagt, let reduceret hos hanunger og pesticidniveauerne i fostrene viste sig at kunne forudsiges vhja. PBK modellering indenfor en faktor 2, hvilket demonstrerer metodens anvendelighed.
Kort fortalt har vi identificeret tre pesticider som værende svage hormonforstyrrende stoffer og har demonstreret anvendeligheden af tilgangen ved en 'proof-of-principle' undersøgelse, der viste sig at kunne forudsige skadelige effekter på det mandlige reproduktionssystem. Dette koncept vil I fremtiden kunne anvendes til at prioritere de 353 pesticider til yderligere in vivo testning.

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