Ministry of Environment
of Denmark
Environmental
Protection Agency

Towards an improved human risk assessment of pesticides for adverse effects on male reproductive health

Pesticide Research
no 210

July 2022

Publisher: The Danish Environmental Protection Agency

Authors:
Scholze M[2],
Taxvig C[1],
Kortenkamp A[2],
Boberg J[1],
Christiansen S[1],
Svingen T[1],
Lauschke K[1],
Frandsen H[1],
Ermler S[2],
Hermann SS[1],
Pedersen M[1],
Lykkeberg AK[1],
Axelstad M[1] & Vinggaard AM[1].
[1]National Food Institute, Technical University of Denmark
[2]Brunel University, UK

ISBN: 978-87-7038-430-8

Preface

The presented project called JANUS (2017-2020) is a collaboration between DTU Food and Brunel University, UK. It is a follow-up project to the 'PANDA' project (Prediction of persistent health effects caused by widely used anti-androgenic pesticides) (2013-2016), in which we initially started development of our QIVIVE approach. The work has been financed and supported by grants from the Danish Environmental Protection Agency, grant no. 667-00153 and 667-00256.

This report is partly based on the content of this publication:
Scholze M, Taxvig C , Kortenkamp A, Boberg J, Christiansen S, Svingen T, Lauschke K, Frandsen H, Ermler S, Hermann SS, Pedersen M, Lykkeberg AK, Axelstad M & Vinggaard AM. *Quantitative In vitro to In vivo Extrapolation (QIVIVE) for predicting reduced anogenital distance produced by anti-androgenic pesticides in a rodent model for male reproductive disorders.* Environ. Health Perspect.,128 (11): 117005, 2020.
In this publication, a major part of the work that has been conducted in this project has been described. Therefore, we have used several paragraphs, figures and tables from this paper in this report, including a reference to the original work.

The authors would like to acknowledge Ulla Hass for scientific advice on the animal studies. We also thank Birgitte Møller Plesning, Heidi Letting, Mette Voigt Jessen, Dorte Lykkegaard Korsbech, Lillian Sztuk, Ulla El-Baroudy, Vibeke Kjær, Sarah Grundt Simonsen, Anne Ørngreen and co-workers at the animal facility at DTU Food for their excellent technical support and assistance.

Contents

1. Summary

Many pesticides can antagonize the androgen receptor (AR) or inhibit androgen synthesis *in vitro* but their potential to cause reproductive and/or developmental toxicity related to the disruption of androgen action in fetal life is difficult to predict. There are currently no golden standard approach for utilizing *in vitro* data to anticipate such *in vivo* effects. Prioritization schemes that limit unnecessary *in vivo* testing are missing altogether.

The aim of the project was to develop a quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) approach that can be used to predict *in vivo* anti-androgenicity arising from exposures in fetal life and manifesting as a shortening of male anogenital distance (AGD) in rats.

We built a physiologically-based pharmacokinetic (PBPK) model to simulate concentrations of test chemicals in the fetal compartment resulting from maternal dosing. The predicted fetal levels were compared with analytically determined concentrations, and in turn these were judged against *in vitro* active concentration ranges for AR antagonism and androgen synthesis suppression.

We first evaluated our model by using data for vinclozolin, linuron, and procymidone for *in vitro* anti-androgenic action and shortened AGD in male pups. Our PBK model described the measured concentrations of parent compounds and metabolites in the fetal compartment quite accurately (within a factor of five). We applied the model to nine current-use pesticides, all with *in vitro* evidence for anti-androgenicity but missing *in vivo* data. Seven pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid and o-phenylphenol) were predicted to produce a shortened AGD in male pups, while two (λ-cyhalothrin and pyrimethanil) were anticipated to be inactive *in vivo*. We tested these expectations for fludioxonil, cyprodinil, dimethomorph and imazalil and observed AGD changes in male rat offspring after exposure during gestation for the first three pesticides. The measured concentrations in the fetal compartments agreed well with the levels predicted by our PBK model. The ratios between observation and prediction were in the range between 0.5 and 2. For imazalil no effect on AGD was observed in contrast to what was expected based on the initial QIVIVE approach. This appeared to be due to the difficulty of the PBK modelling approach to predict accurate internal fetal exposure levels for this compound with complex toxicokinetic features. However, new available kinetic data made it possible to develop an updated PBK model for imazalil, which better predicted the *in vivo* response.

Our QIVIVE model newly identified fludioxonil, cyprodinil and dimethomorph as *in vivo* anti-androgens. With the examples investigated, our approach shows great promise for predicting *in vivo* anti-androgenicity (AGD changes) for chemicals with *in vitro* activity and no unusual toxicokinetic features. It is applicable to agents that produce androgen insufficiency by AR antagonism and/or suppression of androgen synthesis. Our model has the capability to minimize unnecessary *in vivo* testing, but before its potential can be realized fully, further studies with a larger set of chemicals that are not pesticides are required. The limitation is the availability of experimental and/or predicted kinetic data for the chemicals in questions. Thus, if toxicokinetic data are not limited then the approach seems promising for chemicals in general.

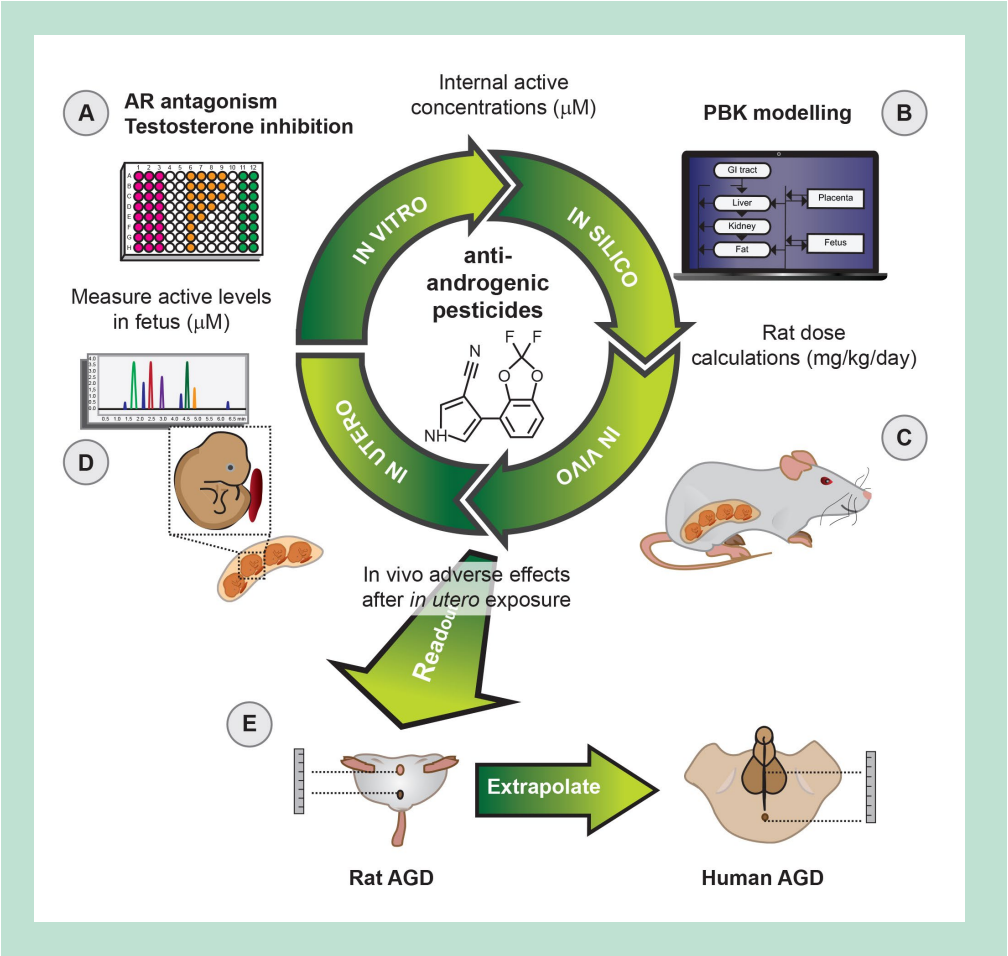


FIGURE 1. Schematic representation of the strategy used to evaluate the anti-androgenic potential of selected pesticides. A) In vitro assays screen for mechanistic endpoints such as androgen receptor (AR) antagonism or inhibition of testosterone synthesis. B) Active In vitro concentration ranges determine the target fetal levels for which the corresponding intake doses are simulated by physiologically-based pharmacokinetics (PBK) models (reverse dosimetry). These are built on in vivo data from existing studies. C) Rats are exposed prenatally at non-toxic doses that are expected to produce shortened anogenital distance (AGD) in male offspring (in vivo study). D) Internal exposure levels are measured in the fetus and dam and compared with simulations outcomes of B. E) Exposed offspring are analyzed morphometrically for AGD. Figure taken from Scholze et al. 2020.

2. Dansk resume

Mange pesticider kan blokere androgenreceptoren (AR) eller hæmme androgen-syntese *in vitro*, men deres potentiale til at forårsage reproduktionstoksicitet og udviklingstoksicitet som følge af forstyrret AR signalering i drengefostret er vanskelig at forudsige uden dyreforsøg. Der findes i øjeblikket ingen metoder til at prædiktere sådanne *in vivo* effekter ved inddragelse af *in vitro* data, og der mangler i det hele taget metoder til at prioritere og begrænse unødvendig *in vivo* testning.
Formålet med dette projekt var at udvikle en kvantitativ metode baseret på ekstrapolation af *in vitro* data til *in vivo* situationen (forkortet QIVIVE), som kan bruges til at prædiktere kemikaliers *in vivo* antiandrogene effekter, der opstår som følge af eksponering i fostertilværelsen og som manifesterer sig som en reduktion af den anogenitale afstand (AGD) i nyfødte hanrotter.

Vi udviklede en fysiologisk-baseret toksikokinetisk (PBK) model til at simulere koncentrationer af testkemikalier i fostret efter oral eksponering af moderen. De prædikterede kemikalie-niveauer i fostret blev sammenlignet med analytisk målte pesticidkoncentrationer, og disse blev til gengæld holdt op imod *in vitro* aktive koncentrationer for AR-antagonisme og androgen-syntesehæmning.
Vi evaluerede først vores model ved hjælp af data for *in vitro* anti-androgen virkning og forkortet AGD hos nyfødte han-fostre for de velkendte antiandrogener vinclozolin, linuron og procymidon. Vores PBK-model forudsagde de målte koncentrationer i fostret af 'parent compounds' og metabolitter relativt nøjagtigt (inden for en faktor fem). Vi anvendte modellen på ni aktuelle pesticider, alle med *in vitro* data for anti-androgen effekt, men hvor der manglede *in vivo* data. Syv pesticider (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid og o-phenylphenol) blev prædikterede at forårsage en forkortet AGD hos hanunger, mens to (λ-cyhalothrin og pyrimethanil) blev forudsagt at være inaktive *in vivo*. Vi testede disse forventninger for fludioxonil, cyprodinil, dimethomorph og imazalil og observerede for de første tre pesticider de forventede AGD-reduktioner i nyfødte hanrotter efter eksponering under drægtighed. De målte pesticidkoncentrationer i fostrene stemte godt overens med niveauet der blev forudsagt af vores PBK-model. For imazalil blev der dog ikke observeret nogen effekt på AGD i modsætning til hvad der var forventet baseret på den indledende QIVIVE-tilgang. Dette viste sig at være på grund af udfordringer ved PBK-modelleringsmetoden med at forudsige en nøjagtig intern eksponering i fostret for dette pesticid, der har en kompleks toksikokinetik. Imidlertid gjorde nye tilgængelige kinetiske data det muligt at udvikle en opdateret PBK-model for imazalil, som bedre forudsagde *in vivo* responset.

Ved hjælp af vores nyudviklede QIVIVE tilgang har vi identificeret fludioxonil, cyprodinil og dimethomorph som *in vivo* anti-androgener. Med de undersøgte eksempler er vores tilgang meget lovende i forhold til at forudsige *in vivo* anti-androgenicitet (AGD-ændringer) for kemikalier med *in vitro* aktivitet og mere traditionelle toksikokinetiske egenskaber. Tilgangen kan anvendes for stoffer, der producerer androgeninsufficiens som følge af AR-antagonisme og hæmning af androgensyntese. Vores model har potentialet til at minimere unødvendige *in vivo* forsøg, men inden dets potentiale kan realiseres fuldt ud, kræves yderligere undersøgelser med et større antal kemikalier.

3. Introduction

Currently approximately 400 pesticides are approved for use in the EU according to the EU Commission Pesticides database (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/active-substances/?event=search.as). Even though criteria for assessing the endocrine disrupting properties of these compounds are now in place within the EU, most of these substances have not yet been evaluated in terms of adverse effects related to endocrine disruption (ED), including those relevant to human male reproductive health. Many current-use pesticides are capable of antagonizing the androgen receptor (AR) or of inhibiting testosterone synthesis *in vitro* (Mnif et al. 2011; Orton et al. 2011; Vinggaard et al. 2002; Vinggaard et al. 2008), but data pertaining to their potential for endocrine disruption *in vivo* are limited or missing altogether. To support such assessments in the future, certain pesticides will need to re-enter targeted developmental and reproductive toxicity studies, in terms of animal numbers and resources the most demanding and costly study format in regulatory toxicology, accounting for no less than 90% of all animals used in regulatory testing (Hartung and Rovida 2009). Until now, no practicable approach exists on how to utilize *in vitro* data to anticipate the potential for *in vivo* reproductive and developmental effects related to the disruption of androgen action in fetal life. As a result, prioritization schemes that can limit unnecessary *in vivo* testing are missing altogether.

Quantitative *in vitro* to *in vivo* extrapolation methods (QIVIVE) have the potential to fill this gap. They hold the promise of delivering urgently needed prioritizations for reproductive and developmental toxicity testing. Interest in such approaches has increased considerably since EU and US regulatory authorities regarded the development of alternative animal-free testing strategies as the most important challenge in future chemical risk assessment (European Commission (EU) 2014; Kavlock et al. 2018). The vision is to mobilize improved mechanism-based toxicological understanding to avoid unnecessary animal testing, and even to replace *in vivo* testing through the use of new approaches including *in vitro* data and in silico methods (Kavlock et al. 2018). To achieve this goal, good mechanistic understanding of the relation between *in vitro* and *in vivo* endpoints is essential. Most promising are QIVIVE approaches, which relate substance concentrations associated with *in vitro* responses to *in vivo* intake doses and their corresponding concentrations in target tissues. QIVIVE methods are derived from mechanistic Physiologically Based Pharmacokinetic (PBK) models that simulate the kinetic dynamics of a substance within the living organism over time (e.g. absorption, distribution, metabolism and excretion). To our knowledge no generally accepted methodology for how to do this exists.

There is considerable interest in QIVIVE models for anti-androgenicity because the incidences of male reproductive disorders such as cryptorchidism, hypospadias, poor semen quality and testicular cancer have risen over the last few decades (Skakkebaek et al. 2016). Human exposure to environmental chemicals, including pesticides, may be among the contributing risk factors, and prenatal or early life exposures are of particular concern (Juul et al. 2014; Swan 2006).

Disruption of androgen action in fetal life by AR antagonists materializes as a syndrome of effects characterized by hypospadias, testes non-descent (cryptorchidism), epididymal lesions, severe prostate lesions, reduced sperm production and shorter anogenital distance (AGD) (Gray et al. 2004). This syndrome is the manifestation of a disturbance of the balance between cell proliferation and apoptosis in androgen-sensitive tissues, which arises from the displacement of androgens from the AR by receptor antagonists. Suppression of steroid synthesis can produce a similar constellation of effects. AGD changes are morphometric biomarkers for adverse male reproductive health outcomes originating during fetal life, both in animals and humans (Thankamony et al. 2016). In rodents and humans, short AGDs in males are strongly associated with adverse male reproductive disorders such as hypospadias, cryptorchidism, and

poor sperm quality (Hsieh et al. 2008; Mendiola et al. 2011; Schwartz et al. 2019; Swan 2006). Current OECD test guidelines for developmental and reproductive toxicity testing regard AGD changes as an adverse outcome that should be considered for estimating no-observed-adverse-effect-levels (OECD 2013).

We present a QIVIVE approach that utilizes *in vitro* AR antagonistic and androgen synthesis-suppressing properties of chemicals to predict dose ranges for which shortened AGDs in male fetuses or pups can be expected in rodent studies after gestational exposures to pesticides. We developed a generic PBPK model structure that simulates internal exposure levels in the fetus at critical time windows after repeated maternal dosing. The quality and reliability of the developed PBK models depends on the needed input data availability and quality for each pesticide. We utilized data in the final Draft Assessment Reports (DAR) for pesticide active substances from the European Food Safety Authority (EFSA) to estimate key kinetic parameters and to identify maximal non-toxic dose limits (EFSA 2015, 2019).

4. Aims and content

We put forward the following hypotheses:

- Anti-androgenic activities determined *in vitro* can predict the internal concentrations required to cause male reproductive disorders in male offspring
- Computational models (i.e. PBK models) can be applied to predict absorption, distribution, metabolism and elimination, as well as fetal concentrations of pesticides with a reasonable certainty
- PBK models, which includes the fetal compartment, can be considered generic and is capable of covering the 'chemical space' of anti-androgens
- The integrated *in vitro* and PBK approach can roughly predict which chemicals that will affect male reproductive organs and can be applied for prioritizing pesticides for *in vivo* testing
- Metabolism can be incorporated into cell assays by a co-culture system and can be applied to inform the PBK modelling.

We addressed the hypotheses by employing a stepwise proof-of-concept approach in which we:

- first evaluated the general applicability of the QIVIVE approach to three well-studied anti-androgenic compounds (vinclozolin, linuron, procymidone) for which *in vitro* and *in vivo* data clearly show anti-androgenic action *in vitro* and shortened AGD in males,
- applied the QIVIVE approach to nine current-use pesticides, all with *in vitro* evidence for anti-androgenicity but missing *in vivo* confirmation, by simulating dose ranges that are expected to produce a shortened AGD in males, and
- selected four of these pesticides for further *in vivo* studies in which we tested our hypothesis that all these substances produce shortened AGD at low non-toxic doses.

The principles of the QIVIVE approach are illustrated in Figure 1.

The various elements of this report have been published in the article by Scholze et al. in the journal Environ Health Perspect 128 (11): 117005, 2020. Therefore, some repetitions cannot be avoided in this report

This report includes:

1) Description of the developed QIVIVE approach. The main methodologies, findings and conclusions are presented in the main text of this report. However, background information and detailed methodologies for various elements in the QIVIVE approach are presented in the Appendices:
    - The detailed PBK modelling procedure described in Appendix 1.
    - *In vitro* information described in Appendix 2.
    - Reports on *in vivo* studies in Appendix 3.
    - Chemical analyses in Appendix 4
    - Overview of experiments performed in PANDA and JANUS in Appendix 5
2) Apart from the QIVIVE approach, additional information includes an evaluation of placental transfer of the pesticides and an evaluation of the possibility of incorporating metabolism into *in vitro* tests.

5. Materials & methods

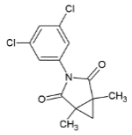
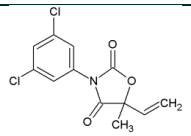
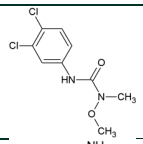
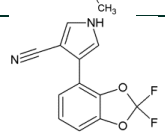
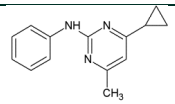
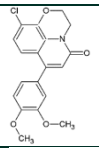
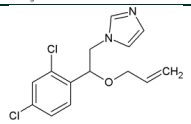
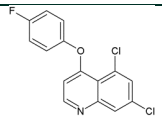
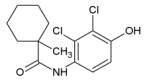
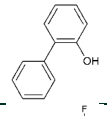
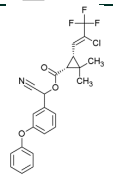
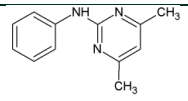
5.1 Chemicals

The chemical structures and suppliers of the test compounds used for *in vitro* and *in vivo* studies are shown in Table 1. Corn oil and dimethyl sulfoxide were used as the vehicle for the *in vivo* and *in vitro* studies, respectively, and purchased from Sigma-Aldrich (Brøndby, Denmark). The positive controls for effects on steroidogenesis, prochloraz (purity 98.5 %, Cas no. 67747-09-5) and forskolin (purity 98%, Cas no. 66575-29-9), were purchased from Dr. Ehrenstofer GmbH (Augsburg, Germany) and Sigma-Aldrich (Brøndby, Denmark), respectively.

5.2 Selection of test compounds

In order to assess the applicability of the QIVIVE approach we selected three model pesticides, vinclozolin, procymidone and linuron, based on their well-known *in vitro* and *in vivo* anti-androgenic effects (Gray et al. 2001; Hass et al. 2007; Orton et al. 2011). Linuron was tested *in vivo* to confirm previously reported changes in AGD following gestational exposure (Ding et al. 2017; McIntyre et al. 2002). In addition, QIVIVE simulations were conducted on nine pesticides currently authorized for use on the European market. We selected these substances according to the following criteria: (1) high relevance in terms of expected exposure to humans (Orton et al. 2011) (2) AR antagonist properties *in vitro* (Orton et al. 2011), and (3) lack of data from *in vivo* studies on male reproductive health. The selected pesticides were: fludioxonil, cyprodinil, dimethomorph, imazalil, fenhexamid, λ-cyhalothrin, quinoxyfen, pyrimethanil, and o-phenylphenol. Of these, four pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil) were chosen for follow-up *in vivo* studies, based on the outcome of our QIVIVE. The selected pesticides, their purity and suppliers are listed in Table 1.
Some experiments mentioned in this report arise from the PANDA project and others from the present JANUS project. An overview of which pesticides that have been investigated in which model and which project is now included in Appendix 5.

TABLE 1. Names and structures of the pesticides included in the project

	Structure	CAS number	Supplier	Purity
Procymidone		32809-16-8	Sigma-Aldrich	99.9 %
Vinclozolin		50471-44-8	Sigma-Aldrich, BOC Sciences[a]	99.6 % 99.5 %
Linuron		330-55-2	Sigma-Aldrich, Greyhound Chromatography & Allied Chem[a]	99.7 % 99.5 %
Fludioxonil		131341-86-1	Sigma-Aldrich	99.9 %
Cyprodinil		121552-61-2	Sigma-Aldrich	98 %
Dimethomorph		110488-70-5	Sigma-Aldrich	> 95 %
Imazalil		35554-44-0	Sigma-Aldrich BOC Sciences[a]	> 99 % >98.5%
Quinoxyfen		124495-18-7	Sigma-Aldrich	> 99 %
Fenhexamid		126833-17-8	Sigma-Aldrich	> 99 %
o-Phenylphenol		90-43-7	Sigma-Aldrich	99 %
λ-Cyhalothrin		91465-08-6	Sigma-Aldrich	> 99 %
Pyrimethanil		53112-28-0	Sigma-Aldrich	> 99 %

[a] Supplier for *in vivo* study

5.3 *In vitro* profiling

5.3.1 Androgen receptor antagonism studies

Vinclozolin, procymidone, linuron and the main metabolites of vinclozolin and linuron were tested for AR antagonistic effects in the AR-EcoScreen assay, an Androgen Receptor Stably Transfected Transcriptional Activation Assay (AR STTA) described in OECD test guideline no. 458 (OECD 2016). Experiments were run with three technical replicates and repeated thrice. The mean value of technical replicates represents one biological replicate. AR-EcoScreenTM cells (JCRB1328, Japanese Collection of Research Bioresources) were grown in CellBIND® Surface cell culture flasks (Corning® Inc., Corning, New York, USA) in growth medium consisting of Gibco® DMEM/F-12 Nutrient Mixture with L-glutamine and HEPES and without phenol red supplemented with 5% FBS, 1% Penicillin-Streptomycin, 200µg/ml ZeocinTM Selection Reagent, and 100µg/ml Hygromycin B (all reagents from InvitrogenTM, Life TechnologiesTM, Carlsbad, California, USA). The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. On the day before the experiment, cells were plated in white 96-well plates (Corning® Inc., Corning, New York, USA) at a density of 9,000 cells/well in assay medium consisting of Gibco® DMEM/F-12 Nutrient Mixture medium, 1% Penicillin-Streptomycin, with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) (all reagents from InvitrogenTM, Life TechnologiesTM, Carlsbad, California, USA). On the following day, the medium was changed to assay medium containing various concentrations of the control compounds R1881 (known AR agonist, Perkin Elmer) or hydroxyflutamide (known AR antagonist, CAS No. 52806-53-8, Toronto Research Chemicals, Toronto, Ontario, Canada). The test compounds were added to the cells in 2-fold serial dilutions ranging from 0.01-3.2 µM for vinclozolin, 0.003-0.8 µM for procymidone, and 0.3-80 µM for linuron. The test concentrations were selected based on concentration-response range finding studies. The DMSO vehicle concentrations were constant in all wells. When testing for AR antagonism, the cells were primed with R1881 at a concentration of 0.1 nM. After cells had been exposed to test compounds for ~20 hours firefly luminescence was measured in a luminometer (LUMIstar® Galaxy, BMG LABTECH, Offenburg, Germany) using Dual-Glo®lLuciferase Reagent from the Dual-Glo® Luciferase Assay System from Promega (Madison, Wisconsin, USA). Cytotoxicity was measured by Renilla luminescence using Dual-Glo® Stop & Glo® Reagent from the Dual-Glo® Luciferase Assay System (Promega, Madison, Wisconsin, USA).

5.3.2 Steroid synthesis in vitro studies

To capture effects on steroid hormone synthesis (e.g. testosterone, androstendione), we tested 12 pesticides (Table 1) in the H295R assay with the human adrenocortical carcinoma cell line NCI-H295R (ATCC no. CRL-2128, LGC Standards, Boras, Sweden) as previously described (Hecker et al. 2011; Rosenmai et al. 2013). Culture medium was removed and stored at -80 °C until the levels of ten steroid hormone were measured using HPLC–MS/MS, as previously described (Rosenmai et al. 2013). Each pesticide including the metabolites of linuron were tested at seven concentrations in three independent experiments, and hormone levels were normalized to the solvent controls containing 0.1% DMSO. Cytotoxicity was evaluated by using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (cat.no. M2128, Sigma, St. Louis, US) as previously described (Hecker et al. 2011; Rosenmai et al. 2013). Fluorescence was measured (excitation 560 nm, emission 590 nm) using a Wallac Victor2 1420 multilabel counter (PerkinElmer, Massachusetts, US).

5.3.3 Incorporation of metabolism into *in vitro* methods

For some pesticides it is the metabolites that are the active anti-androgenic compounds. Therefore it is essential to have incorporated metabolism into the *in vitro* experiments in order to predict *in vivo* responses. In this project we continued the work done in the InVita project

(MST 667-00190; Taxvig, 2020) concerning inclusion of metabolism in *in vitro* assays. We evaluated the effects of incorporating a metabolising system based on rat liver enzymes (S9) in the two *in vitro* assays for endocrine disruption (the AR EcoScreen and the H295R assay) used in this project, based on the standard protocol for the respective assays (OECD, 2016 and OECD, 2011). In addition, we incorporated a metabolising step, in which selected compounds were pre-incubated with rat liver S9 mix prior to standard testing in the assays. The S9 liver fraction primarily include Phase I liver enzymes. The focus was directed towards Phase I metabolism, as Phase II enzymes are expected to mainly result in formation of metabolites with decreased activity compared to the parent compound.
For the S9 incubations a 100X (10mM) stock of each of the test compounds was prepared in acetonitrile. The incubation reactions were performed in sterile glass test tube containing a final concentration of: 84 mM Phosphate buffer, 2.0 mg/mL rat S9, 100µM test compound, and 1 mM NADPH-RS (NADPH Regenerating System from Xeno Tech).
The glass test tubes were placed into a 37°C shaking water bath, and the samples were incubated for 60 min. After incubation the enzyme reaction was stopped by adding 5x sample volume of cold methanol. The samples were centrifuged at 16,000g at 4°C for 5 min and the supernatants were transferred to clearly labelled HPLC vials and store at -20°C until further use. At the day of exposure, the methanol was evaporated from the samples and the compound+S9 sample was dissolved in the respective assay media and added to the cells (Taxvig, 2020). The AR-Eco Screen and the H295R assay were run as previously described in Rosenmai et al. 2021 and Rosenmai et al. 2013.

5.4 PBK modeling

A generic PBK model for rodents with emphasis on the physiological and biochemical changes that occur during gestation after repeated daily oral dosing of environmental compounds was adopted from previously established gestational PBK models (Emond et al. 2006; O'Flaherty et al. 1992). The focus of our flow-limited model was on the simulation of internal pesticide concentrations in the blood plasma of the fetus at gestational day (GD) 15 to 18, a developmental stage, which is considered as most critical for male sexual differentiation in rats (Sharpe 2006). The model structure was kept parsimonious and included only maternal tissues and kinetic processes that were considered as directly relevant for the estimation of fetal exposure levels (Figure 2): liver and kidney as the major sites of elimination and metabolism, fat tissue to account for potential lipophilicity, blood/plasma for the description of the systemic circulation, and two remaining compartments that include all other well- or poorly-perfused organs and tissues lumped together for calculation of the mass balance.

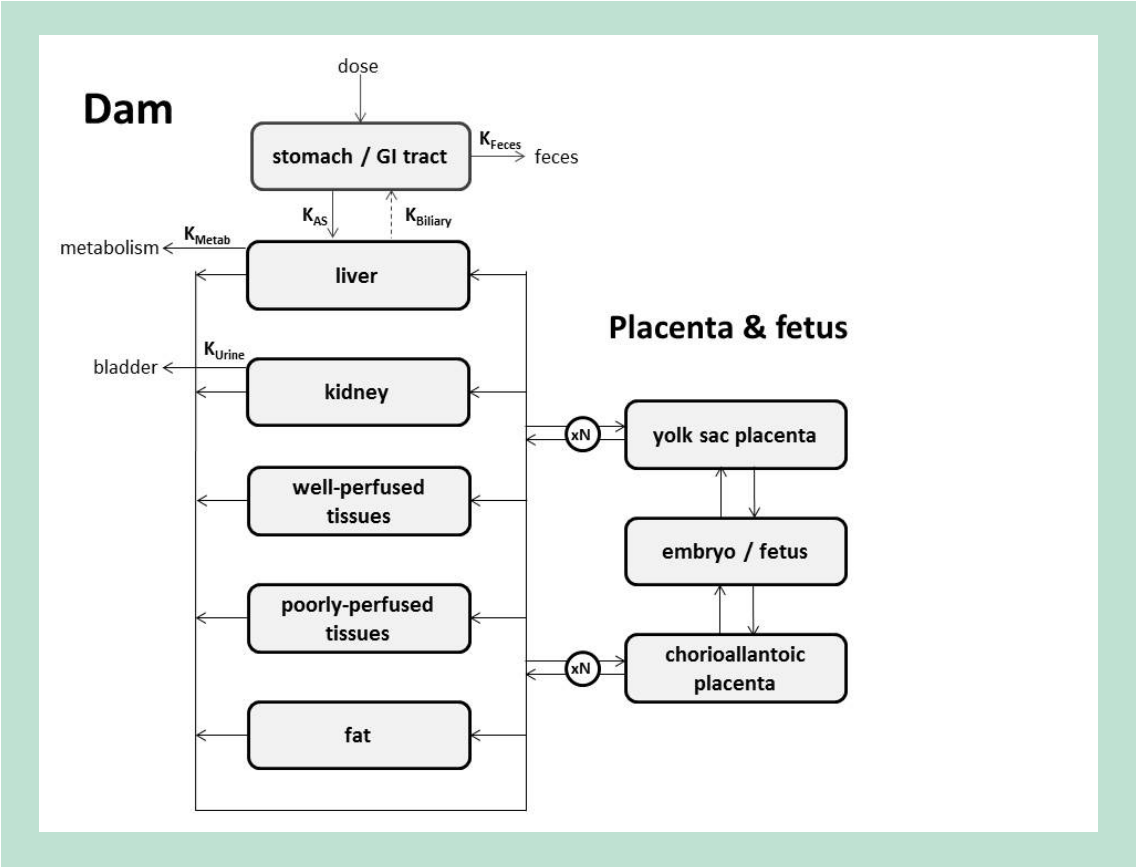


FIGURE 2. Structure of the PBK model for gestational exposure in rats. This physiologically-based kinetic (PBK) model was adopted from (O'Flaherty et al. 1992) with several physiological modifications suggested by (Emond et al. 2006). Figure was taken from Scholze et al. (2020). 'xN' stands for the number of concepti.

As all model compounds were non-volatile, the concentration of chemicals in venous blood was assumed to be equal to its concentration in arterial blood; therefore, a lung compartment was not included. The active uptake into the gastrointestinal (GI) tract and the absorption from the GI tract into the liver were 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 was modeled as a simple clearance rate from the liver to the intestine (enterohepatic recirculation) with no time delay. The latter was considered as a viable model option as (1) some pesticides are conjugated with a molecular weight above 325, which is often considered as lower bound for the molecular weight for enterohepatic circulation in the rat (LeBlanc and Hodgson 1987), and (2) to allow more model flexibility. This set of maternal compartments was considered as sufficient to estimate and calibrate kinetic elimination model parameters from *in vivo* kinetic data. The model structure was extended by two placental units (yolk sac and chorioallantoic placenta) and the fetal compartment. For model simplicity, the whole fetus was implemented as a single diffusion-limited compartment with no further division into individual tissues or compartments, where the bi-directional transfer from the placenta units to the embryo/fetus and vice versa was described by first-order diffusion rates (activated on GD6). Elimination processes in the fetus were not considered, and transfers between fetal levels and amniotic fluid were not included in the model structure. The fetal plasma concentrations were approximated as the average concentrations over the time interval between GD15-18 and estimated as the area under the curve (AUC) divided by 72 hrs (AUCfetus). Similarly, a maternal concentration at GD21 was defined as the average level in the circulating blood system over the time between the last feeding and birth and calculated as AUC divided by 24 hrs.

Physiological model parameters were retrieved from reference-values for laboratory animals and literature, pesticide-specific kinetic parameters (log Kow, plasma binding) were determined from in silico methods when no experimental data were available, absorption and elimination model parameters were estimated directly from kinetic *in vivo* data as reported in EFSA DARs (EFSA 2015) or our own studies (procymidone, vinclozolin, linuron), and tissue-partitioning of pesticides was found in DARs or estimated according to (Poulin and Theil 2002). The bi-directional transfer between placenta compartments and fetus was described by a worst-case range of first-order diffusion rates. The PBK model was implemented in Berkeley Madonna (software version 8.3.18); All data and equations for PBK modeling is provided in Appendix 1.

5.5 QIVIVE

PBK simulations were used to produce an *in vitro-in vivo* profile for the most likely internal exposure concentration in the fetal compartment at a given intake dose and time. We defined a dose window for potential anti-androgenic effects *in vivo* whose lower and upper limits, respectively, were demarcated by (i) the dose expected to produce fetal levels equivalent to *in vitro* concentrations associated with at least 20% AR antagonistic activity and/or testosterone inhibition, and (ii) the dose anticipated to be below a range associated with maternal or pre-natal toxicity. A 20% inhibition of AR antagonism was judged to reflect a lowest effect level *in vitro*. This upper dose was derived from data reported in one- or two-generation developmental and reproductive toxicity studies and selected as falling between the highest dose without any observed adverse effects (usually the no-observed-adverse-effect) and the lowest dose at which an adverse effect materialized (the lowest-observed-adverse-effect). All toxicity descriptors that were used to define the anticipated dose range for anti-androgenicity for the pesticides can be found in Table 3 and Appendix 2.

5.6 *In vivo* reproductive/developmental toxicity studies

In total five main *in vivo* exposure studies were conducted with exposures to pregnant rats during gestation and lactation to the selected pesticide (Table 2). An overview of the project in which the various experiments were conducted are given in Appendix 5. Firstly, a selected dose from procymidone and vinclozolin was tested to measure internal body concentrations in the dams and fetus shortly before birth (GD21). Secondly, linuron was tested to investigate its internal concentrations, and, as the substance has never been tested in-house before, AGD was assessed in the offspring in order to confirm external outcomes. Thirdly, fludioxonil, cyprodinil and dimethomorph were tested to investigate their effects in male offspring and fetuses. For one selected dose of each compound the internal body concentrations in dams and fetuses were determined by Caesarean section at GD21. Due to the study outcomes for effects on anogenital distance from the third study, we conducted a fourth follow-up study on dimethomorph. Finally, we performed an *in vivo* study on imazalil.

TABLE 2. Overview of *in vivo* rat studies

In vivo study no.	Chemical	Doses (mg/kg bw/day)	Rat strain[a]	Exposure period (dams)[b]	No. of litters/group (no. of controls)	Internal exposures of dams and fetuses
1	Procymidone	40	SD	GD7-21	6 (6)	GD 21
	Vinclozolin	40	SD	GD7-21	6 (6)	GD 21
2[c]	Linuron	25, 50, (75[d])	SD	GD13-21[e] & PD 1-15	9-11 (10)	--
	Linuron	25, 50, (75[d])	SD	GD13-21[e]	3 (3)	GD 21
3[c]	Fludioxonil	20, 60, 180	W	GD7-21 & PD1-17	6-10 (8)	--
	Cyprodinil	20, 60, 180	W	GD7-21 & PD1-17	6-10 (8)	--
	Dimethomorph	6.7, 20, 60	W	GD7-21 & PD1-17	6-10 (8)	--
	Fludioxonil	60	W	GD7-21	2 (1)	GD 21
	Cyprodinil	60	W	GD7-21	2 (1)	GD 21
	Dimethomorph	20	W	GD7-21	2 (1)	GD 21
4[c]	Dimethomorph	6.7, 20, 60, 180	SD	GD7-21 & PD1-16	10-11 (11)	--
5	Imazalil	8, 24, 72	SD	GD7-21 & PD1-16	14-16	GD21

a) W: Wistar rats; SD: Sprague Dawley rats; b) GD: gestation day, PD: postnatal day; c) These studies were modified OECD TG 421 studies (Reproduction/Developmental Toxicity Screening Test) (OECD 2016a); d) Maternal toxicity was observed at the highest dose and for this reason only results from the 25 and 50 mg/kg bw/day doses are included for postnatal endpoints in this report; e) different exposure period in order to reduce maternal toxicity; Details on study design and obtained results for study 2, 3, 4, and 5 can be found in Appendix 3. This table is modified from a table presented by Scholze et al.(2020.

5.7 Exposure and study design

The study with procymidone and vinclozolin was conducted with Sprague-Dawley rats, NTac:SD strain, (SPF, Taconic Europe, Ejby, Denmark) (study 1). For the *in vivo* study with linuron (study 2) we used Sprague-Dawley rats (CD IGS Rat, Crl:CD(SD), Charles River Laboratories, Sandhofer Weg 7, Sulzfeld, Germany). The study comprising fludioxonil, cyprodinil and dimethomorph (study 3) was conducted with time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) as part of the PANDA project. Finally, the *in vivo* studies with dimethomorph (study 4) and imazalil (study 5) were carried out with Sprague-Dawley rats (CD IGS Rat, Crl:CD(SD), Charles River Laboratories. The reason for the use of different rat strains was because of All studies were performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and protocols were approved by the DTU in-house Animal Welfare Committee. The linuron, dimethomorph and imazalil *in vivo* studies were conducted in the animal facilities in DTU Food, Lyngby, whereas all other animal studies were carried out at the DTU animal facility in Mørkhøj, Denmark. Time-mated dams were delivered on gestation day 3 (GD3). On GD4, dams were distributed into exposure groups with similar body weight distributions. Animals were housed in pairs until GD17 and thereafter individually. The animals were kept under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15x27x43 cm) with Aspen wood chip

bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden, Lynge, Denmark). They were placed in an animal room with controlled environmental conditions: 12 hr light-dark cycles with light starting at 9 pm, temperature 22 ± 1°C, humidity 55 ± 5%, 10 air changes per hr. Solutions of all test chemicals were prepared in corn oil and dams were dosed by oral gavage during the morning hours from GD7 to GD21 and from the day after birth to pup day (PD) 16 (the day of expected birth was designated PD1). In the linuron study, exposure of the dams was limited to GD13-21 as maternal toxicity during early gestation has been described previously (McIntyre et al. 2000). There were four blocks of time-mated rats, divided to a control group and three dose groups (25, 50 or 100 mg/kg/day). Maternal toxicity was observed at the highest dose and for this reason only results from the 25 and 50 mg/kg/day doses are included for postnatal endpoints (N=10-11). Further details on this study can be found in Appendix 3.
For the fludioxonil, cyprodinil and dimethomorph studies, 102 time-mated dams in three blocks were divided to dose groups with even body weight distribution. Dams were dosed by gavage with vehicle control (corn oil) or one of the following doses: dimethomorph 6.7, 20, 60 mg/kg/day, cyprodinil 20, 60, 180 mg/kg/day, and fludioxonil 20, 60, 180 mg/kg/day (N=10-12). In the follow-up dimethomorph study, 60 time-mated dams were dosed by gavage with vehicle control (corn oil) or one of the following doses: 6.7, 20, 60 and 180 mg/kg/day. Results on AGD can be found in the main part of this report, while other information can be found in Appendix 3.
In addition a range finding study and a large dose response study (including GD 21 sections) on imazalil was part of this project (see Appendix 3). In brief, dams were dosed by oral gavage during the morning hours from GD7 to GD21 and – in studies continuing after birth - again from the day after birth until the pups reached day (PD) 16. Doses of 0, 8 and 24 mg(kg bw/day were selected for the main study due to dystocia and maternal toxicity in the higher dose groups of the range-finding study, and an additional dose of 72 mg/kg bw/day was included as part of the GD 21 cohort only. 80 time mated SD rats, divided into three blocks, were exposed to vehicle or two doses of imazalil (0; 8; 24 mg/kg bw/day) by oral gavage from gestational days (GD) 7 to postnatal day (PND) 16 (N=14-16).

Generally the same study design was applied to all chemicals based on dosing from GD7-GD21 (covering the sensitive male programming window) and when pups were delivered from PD1-16/17. However, exposure to linuron started first at GD13 due to maternal toxicity that arises when exposure starts earlier. The varying animal strains used were due to changes in available strains from the supplier and not a wish from our side.

Pup weights and AGD were measured after birth at PD1 using a stereomicroscope with a micrometer eyepiece and were determined as the distance between the genital papilla and the anus. To discriminate between an anti-androgenic effect and stunted growth, the AGD index (AGDi) was calculated by dividing AGD by the cube root of the body weight (Hass et al. 2007). Apart from effects on AGD also effects on nipple retention were measured in the pups, although these data were not included In the QIVIVE approach. On PD13-14, all male and female pups were weighed and examined for the number of areolas/nipples, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Usually, female rats have 12-13 nipples whereas male rats have none. All measurements were performed blinded with respect to treatment group by the same, skilled technician.

5.8 Fetal and maternal chemical exposure analysis

Caesarean sections were performed at GD21 on additional dams from the following dose groups: 60 mg/kg/day fludioxonil, 60 mg/kg/day cyprodinil, 20 mg/kg/day dimethomorph (N=2 litters), 25, 50, and 75 mg/kg/day linuron (N=3 litters), procymidone and vinclozolin (40 mg/kg bw/day both groups, N=6 litters), 8, 24, 72 mg/kg/day imazalil, N=5). Blood from the dams was

collected and plasma and amniotic fluid frozen directly. Pooled trunk blood from all female and male fetuses of the same litter was collected into heparin-treated capillary tubes approx. 90 min after dosing and plasma prepared and stored at −80 °C until analysis for pesticides and their metabolites by LC-QTOF. Details and descriptions of the pesticide analyses are provided in Appendix 4.

5.9 Statistics

For the analyses of *in vivo* data, the litter was the statistical unit. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. AGD was analyzed using body weight as covariate, and birth weights were analyzed using the number of offspring per litter as covariate. Dose-related effects on continuous endpoints were analyzed by ANOVA methods (mixed effect modelling), and statistical significance was assessed using multiple contrast tests. The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and 12, with the latter assumed to reflect the biologically possible maximal number of nipples in rats. Litter effects on nipple retention and over-dispersion in the data were accounted for by using Generalized Estimating Equations as reported in (Christiansen et al. 2012). Statistical significance was judged for p-values below the false-positive rate α= 5%. Dose-response regression analysis on all *in vivo* and *in vitro* endpoints was performed by a best-fit approach (Scholze et al. 2001). All statistical analyses were conducted in SAS (SAS Enterprise Guide 4.3) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

5.10 Placental transfer of pesticides

The original plan of the project was to perform the BeWo assay in order to evaluate placental transfer of the pesticides. However, we realized that using the *in vivo* exposure data we had generated on both dams and pups would give us an estimate of the placental transfer that were as least as good as the data from the BeWo assay. Thus, we changed plans and in order to estimate the degree to which the pesticides were able to be transferred from the mother to the fetus, we used the internal exposure levels measured in corresponding dams and foetuses (see Appendix 4 for the method description). In the *in vivo* studies amniotic fluid as well as plasma from both dams and foetuses were collected for chemical analysis in order to measure the levels of parent compound and for two compounds, vinclozolin and linuron, also 2-3 metabolites. We used these data to evaluate the placental transfer of the pesticides in the rat.

Using an average total blood volume (TBV) of 64 ml of blood per kg body weight (BW) for a rat (Lee and Blaufox 1985) and an amniotic fluid volume (AFV) of 0.4 ml at GD21 in rats (Wlodek et al. 2000), we calculated an estimated total placental transfer (ETPT) using the following parameters:

TBV (ml) = 64 ml/kg * BW(kg) Eq. 1
nmol compound in plasma = nM in plasma (from the chemical analysis) * TBV (L) Eq. 2
nmol in amniotic fluid = nM in amniotic fluid (from the chemical analysis) * AFV (L) Eq. 3

$$\text{ETPT (\%)} = \frac{\text{nmol in male+female foetuses + amniotic fluid}}{\text{nmol compound in dam}} * 100\% \quad \text{Eq. 4}$$

We included both the amounts of pesticide in the fetal plasma as well as those in the amniotic fluid to determine the total placental transfer.

6. Results

6.1 Applicability of the QIVIVE approach to three pesticides with known anti-androgenic properties

As compounds with a well-documented ability to induce shortened AGD in male rodent offspring, the pesticides procymidone, vinclozolin and linuron (Gray et al. 2001; Hass et al. 2007) were selected to investigate the general applicability of our QIVIVE approach. First, we tested the three pesticides *in vitro* for AR antagonism and for suppression of androgen synthesis. We were able to confirm that vinclozolin acts primarily by antagonizing the AR (Molina-Molina et al. 2006), with negligible activity in terms of suppressing steroid synthesis. In contrast, procymidone and linuron both antagonized the AR and inhibited the synthesis of androgens such as testosterone and androstendione (Fig 3 & Table 3). In both cases, AR antagonism became apparent at approximately 10-fold lower concentrations when compared to steroid synthesis inhibition.

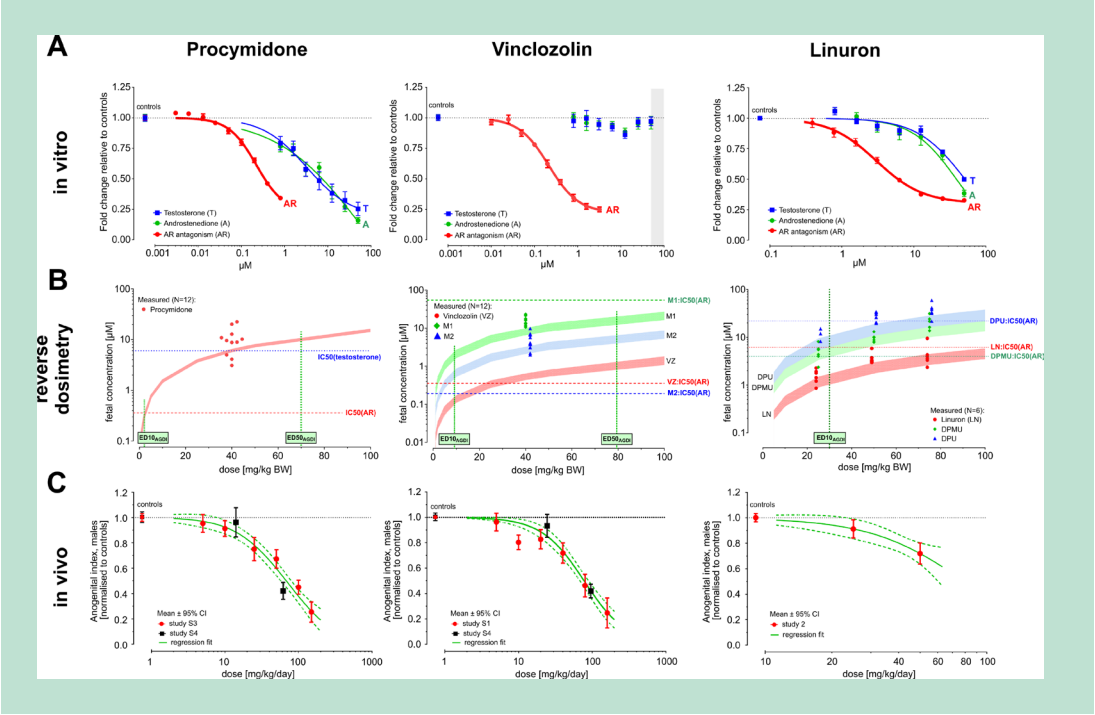


FIGURE 3. QIVIVE for shortened AGD in male offspring following gestational exposure to procymidone, vinclozolin, and linuron. A) Concentration response data *in vitro* and regression curves for AR antagonism (red symbols and lines) as well as testosterone (blue symbols and lines) and androstendione inhibition (green symbols and lines). Data represent mean ± SEM (N = 9), the shaded grey area for vinclozolin indicates cytotoxic concentration ranges in the H295R assay. B) PBK modeled relationships between fetal plasma concentrations of the parent compounds and their relevant metabolites and doses administered to dams. Symbols (●♦▲symbols) show the measured fetal plasma levels in the *in vivo* studies on the 3 compounds. PBK simulations for GD15-GD18 are shown as shaded areas (red for parent compound, blue and green areas for metabolites). The parent compound, vinclozolin, could not be detected in blood or amniotic fluid and only the M1 and M2 metabolites could be quantified. We refer to Table 3 for names of all metabolites included. The shadings reflect least- and worst-case kinetic model assumptions for describing the exposure transport between placenta

and fetal compartment. Horizontal lines show the concentrations associated with strong *in vitro* activities (IC50), vertical lines show doses (ED10, ED50) that resulted in weak and strong reductions of the AGD, respectively, measured in male rat pups (derived from the data shown in C). C) Dose-response data and regression curve for AGD measured in rat male pups shortly after birth following exposure (GD7 to GD21) to procymidone and vinclozolin illustrated with data from our previous study (Hass et al. 2007), as well as to linuron (study 2 of Table 2). AGDI were normalized to the mean AGD response from male controls (set to 1) and female controls (set to 0). Data represent mean ± 95% confidence belt (n = 6-13 litters), the regression curves are mean ± 95% confidence belt. Figure is taken from Scholze et al. 2020.

Next, we measured the levels of the parental compounds and their *in vitro* active metabolites in the blood of fetuses resulting from dosing the dams daily during GD7 – GD21 with 40 mg/kg/day procymidone, 40 mg/kg/day vinclozolin or three doses of linuron (25, 50 and 75 mg/kg/day), all by gavage. The samples were taken on GD21. At this time point, we also determined the concentrations of all compounds (and their metabolites) in the blood of the dams and in amniotic fluid (Figure 4, Table 3). These measurements were used to (i) inform the PBK model in terms of the metabolites to be included in the simulations, and (ii) to estimate kinetic model parameters (together with literature data or in silico methods, Appendix 1). The linuron metabolite 3,4-dichloroanaline (DCA) was not detected in any of the fetuses. For this reason, it was not integrated in the PBK simulations. In the animals exposed to vinclozolin, only the metabolites 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), but not the parent compound, were detected. In both sexes, the plasma levels of these pesticides and their metabolites were similar (Figure 4).

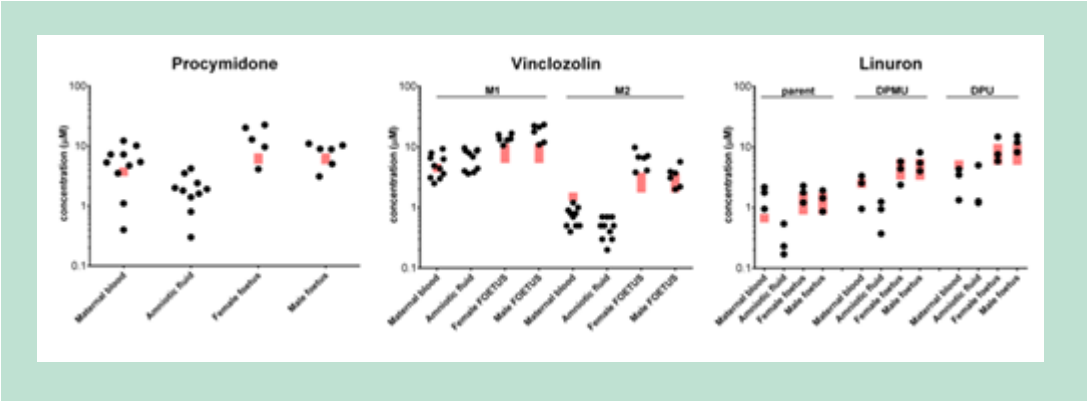


FIGURE 4. Measured and physiologically-based kinetic (PBK)-model-simulated pesticide concentrations in maternal and fetal plasma as well as amniotic fluid after repeated dosing of procymidone (40mg/kg/bw), vinclozolin (40mg/kg/bw), and linuron (25mg/kg/bw), (GD7 - GD21). For vinclozolin, the levels of metabolites M1 and M2 are shown, for linuron those of DPMU and DPU (we refer to Table 3 for the full names of the metabolites). The ranges of simulated concentrations are shown as red bars and refer to average plasma concentrations on gestational day (GD) 21 (dams) or fetal concentrations on GD15 - GD18. Measurements are shown as black dots representing one dam (maternal blood), one litter (amniotic fluid) or gender-specific litter means (fetus) and were taken on GD21 approximately 90 min after dosing by oral gavage. Figure taken from Scholze et al. 2020.

We determined AGD changes in all pups (PND1) exposed to linuron and confirmed its previously reported anti-androgenicity (Gray et al. 2001; McIntyre et al. 2002): 50mg/kg/day linuron caused a 28% reduced AGD in male offspring when compared to female offspring, statistically significantly different from untreated controls (Figure 3C). AGD changes at higher linuron doses were strongly confounded by maternal toxicity and are not shown here. In extensive

dose-response studies conducted previously, we have shown that both procymidone and vinclozolin produced shortened AGDs in male offspring close to values seen in control female pups (Hass et al. 2007) (Figure 3C).
Next, we performed PBK model simulations for a range of doses that included those associated with 10% and 50% reductions in AGD (ED10 and ED50, respectively). This included the parent compounds and their active metabolites. The maternal doses, which we used to determine internal tissue concentrations, fell within the modelled range. Figure 3B shows the concentrations in the fetal compartment predicted to result from these doses, together with the range of measured concentrations. Figure 4 complements these data by also showing predicted and observed levels in the blood of dams and in amniotic fluid.
In most cases, the individual measurements were close to the range of the simulated concentrations by PBK modeling (at least < factor 5). To a certain degree, the good agreement between simulation and observation was due to the fact that we had to utilize the outcome of tissue concentration measurements for the estimation of certain PBK parameters. However, the fetal levels had to be measured at a slightly different time point (GD21) than the simulations (GD15-GD18). Furthermore, the measurements are snapshots of internal exposures for specific time points, while the simulations give values averaged over longer time periods. Overall, we judged the agreement between simulation and observation as satisfactory and saw no need to modify the PBK model structure.

Strikingly, both the simulated and the measured fetal compartment levels that resulted from doses associated with AGD changes (ED10 and ED50) overlapped with the concentration ranges associated with *in vitro* AR antagonism and suppression of steroid synthesis (horizontal lines depicting *in vitro* IC50 values in Figure 3B). For all three compounds, concentrations equivalent to the fetal levels at the ED10 for AGD changes produced almost saturating *in vitro* AR antagonistic effect concentrations. This suggests that there are factors at play *in vivo* that lead to an apparent diminution of the compounds' activity. We speculate that differences in the active, free concentrations are a possible explanation, due to differences in protein concentrations between the cell culture media and the blood plasma.
Overall, our results confirmed our presumption that *in vitro* active concentration ranges, combined with PBK simulations can be used to predict whether active concentrations can be attained in the fetus and whether these in turn are associated with *in vivo* outcomes. This encouraged us to utilize this approach for predictive assessments of compounds not previously tested *in vivo*.

6.2 QIVIVE as a prediction tool

To evaluate the robustness of our QIVIVE approach, we selected nine pesticides currently authorized for use in the EU. We previously identified all these compounds as AR antagonists ((Orton et al. 2011), see Appendix 2 for a summary of potencies), but information about *in vivo* activity was missing, as were data about their capacity to suppress androgen synthesis. To expand the *in vitro* basis of our QIVIVE, we tested all these pesticides for effects on steroid hormone synthesis in the H295R steroidogenesis assay. Seven of our chosen pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid and o-phenylphenol) inhibited testosterone synthesis as well as its precursor androstendione. Fludioxonil and imazalil showed significant activity already at the lowest tested concentration of 0.8 µM (Appendix 2). Two pesticides, λ-cyhalothrin and pyrimethanil, had no effect on any measured androgen. This potency of cyprodinil, imazalil and quinoxyfen in suppressing steroid synthesis exceeded that in antagonizing the AR and vice versa for the remaining four compounds (Figure 5, horizontal lines, Table 3).

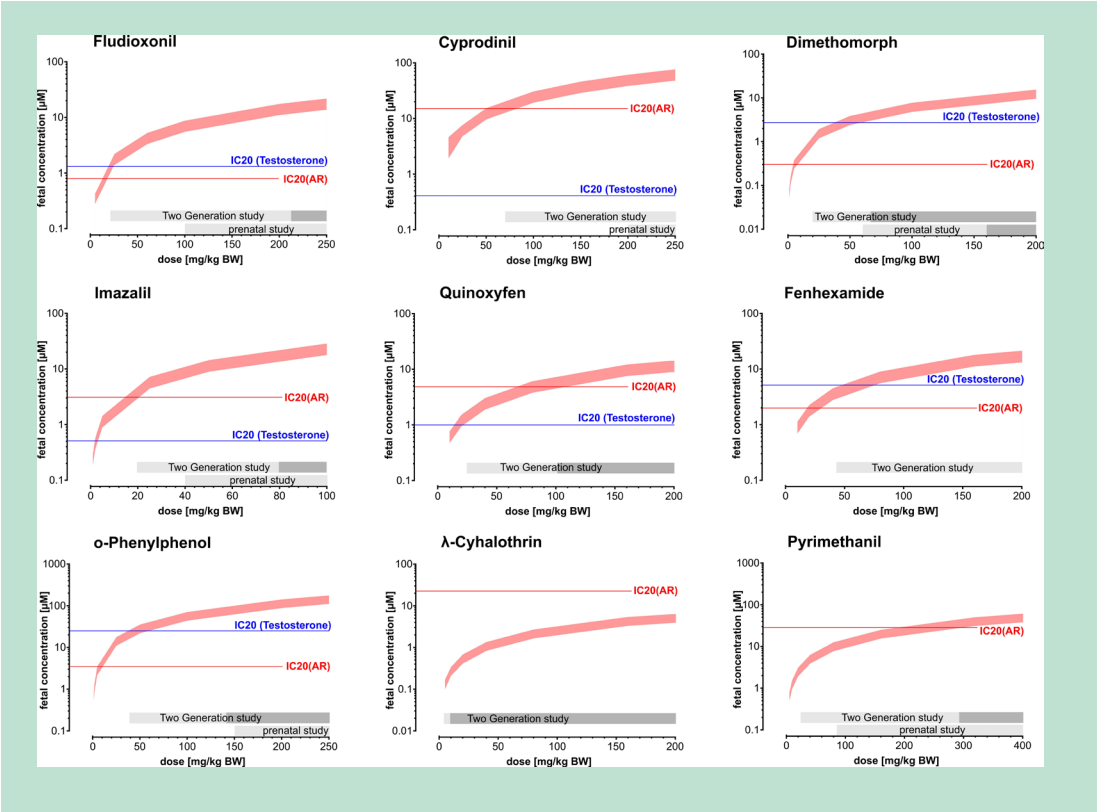


FIGURE 5. Physiologically-based kinetic (PBK) simulations of fetal concentrations of fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, o-phenylphenol, λ-cyhalothrin and pyrimethanil, in response to repeated maternal doses between gestational day (GD) 7 and GD21, depicted as red shaded areas. The red shadings reflect different kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see Materials and Methods and Appendix 1). Horizontal lines indicate the IC20 for AR antagonism *in vitro* (AR) (red line) and the IC20 for testosterone inhibition (blue line) *in vitro*. Grey horizontal bars show dose ranges associated with *in vivo* adverse effects reported in two-generation or prenatal toxicity studies in EFSA's Draft Assessment Reports (OECD TG 416 or TG 414, respectively), with the light grey bar indicating the range between the No Observed Adverse Effect Level (NOAEL) and Lowest Observed Adverse Effect Level (LOAEL) and the dark grey bar indicating the dose range above the LOAELs (NOAEL and LOAEL for λ-cyhalothrin were chosen from studies on cyhalothrin). Figure taken from Scholze et al. 2020.

In order to pinpoint candidates for further *in vivo* investigation, we used the PBK model to simulate fetal concentrations for a wide range of doses (Figure 5). There was no evidence for potential AR antagonist activity or impacts on steroid hormone synthesis for any of the pesticide metabolites. We therefore limited the simulations to the parent compounds.
In order to perform the QIVIVE and predict in vivo intake doses based on the *in vitro/in silico* predictions, one has to investigate figure 5 in detail and look at the points where the horizontal lines illustrating *in vitro* activitites cross the PBK modelling range. From this point, the *in vivo* dose that would be predicted to cause *in vivo* activity can be read at the X axis.
A compound was considered as a candidate for potential anti-androgenic effects in the male rat if (i) doses to the dam were predicted to result in fetal compartment levels equivalent to *in vitro* concentrations associated with at least 20% AR antagonist activity and/or testosterone inhibition, and (ii) if these doses were below the LOAELs reported from 1- or 2-generation reproductive toxicity studies and were not associated with maternal and prenatal toxicity.

Based on these criteria, we expected seven pesticides to induce shortened male AGD *in vivo*: fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, and o-phenylphenol.

Because AR antagonism was not observed at the fetal levels attained after maternal dosing at levels below the toxic range, we anticipated that λ-cyhalothrin and pyrimethanil should not affect AGD at doses below the maternal toxicity threshold (Figure 5).

To test our predictions, we selected fludioxonil, cyprodinil, dimethomorph and imazalil for *in vivo* studies. Fludioxonil antagonizes the AR and inhibits testosterone synthesis with similar potencies. Cyprodinil and imazalil are more potent in suppressing testosterone synthesis than in antagonizing the AR, while the reverse is true for dimethomorph (Figure 7A and Figure 5). The absorption, distribution, metabolism, excretion (ADME) *in vivo* data for fludioxonil, cyprodinil, dimethomorph reported in DARs and other sources were considered as sufficient to estimate the compound-specific first-order elimination rates for metabolism, renal clearance and excretion. However, this was less clear for imazalil as such data were not available for time points earlier than 12 hours after dosing (Mannens et al., 1993), and as consequence not only the estimation of the elimination rates have to be considered as highly uncertain but also the PBK simulations. Furthermore, imazalil undergoes many different routes of metabolism, including epoxidation, epoxide hydration, oxidative O-dealkylation, imidazole oxidation and scission, and oxidative N-dealkylation, with 24 hours after dosing at least 25 metabolites having been discovered in urine and feces (DAR Imazalil Annex B.5). Due to this complex metabolism profile imazalil was selected as "worst-case" in order to test the QIVIVE approach, and outcomes are reported separately from the fludioxonil, cyprodinil and dimethomorph assessment.

TABLE 3. Measured (GD21) and simulated fetal plasma levels (GD15 - GD18) of parent compounds and metabolites after gestational exposure to individual doses of six pesticides, together with AGD responses in male offspring (PND1 or GD 21) and results from *in vitro* profiling for anti-androgenic effects (IC_{50} for AR antagonism and testosterone inhibition).

Compound	*In vitro*		*In vivo*			*In silico*
	AR antagonism (IC_{50}, µM)	Testosterone inhibition (IC_{50}, µM)	Dose (mg/kg/day)	AGD reduction [c] (%)	Measured concentration in male fetus [d] (means in µM)	Simulated concentration in male fetus [e] (range in µM)
Procymidone	0.4	6	40	34[f]	7.8	5.9 – 6.4
Vinclozolin	0.3	n.a.	40	26[f]	< 0.15[g]	0.43 – 0.64
M1	2.0 [a]	n.d.			18.0	9.0 – 14.4
M2	0.2 [a]	n.d.			3.4	0.2 – 0.36
Linuron	6	27	25	9	1.6	0.9 – 1.5
DPMU	4	n.a.			5.0	3.4 – 5.4
DPU	22	5			11.8	6.0 – 9.5
DCA	12	n.d.			n.d.	n.d.
Linuron	6	27	50	28	3.7	1.8 – 3.0
DPMU	4	n.a.			9.6	6.8 – 10.9
DPU	22	5			27.4	12.0 – 19.0
DCA	12	n.d.			n.d.	n.d.
Linuron	6	27	75	n.d.[h]	4.5	2.9 – 4.8
DPMU	4	n.a.			21.4	10.9 – 17.3
DPU	22	5			37.8	19.2 – 30.4
DCA	12	n.d.			n.d.	n.d.
Fludioxonil	2.6[b]	10.8	60	13	3.3	3.3 – 5.4
Cyprodinil	28[b]	8.5	60	16	6.6	11.5 – 18.3
Dimethomorph	0.9[b]	>50	20	13	1.3	1.0 – 1.5

Imazalil	8.2[b]	1.2	24	-1,2%	0.3		4.4 – 7.2[i] 0.2 – 0.31[j]

[a] Our own unpublished data; [b] Orton et al. 2011; [c] In males, normalised to the mean AGDI response from male and female controls (females set to zero); [d] Fetal blood was pooled per litter from all males at GD21 and median litter value is reported; [e] Simulations refer to average plasma levels at GD15 - GD18; kinetic model parameters estimated from *in vivo* studies in EFSA's Draft Assessment Reports;; [f] Estimated from regression model (Hass et al 2007); [g] below limit of quantification; [h] Maternal toxicity; [i] kinetic model parameters provided by EUROMIX; n.a. = no activity detected up to 50µM; n.d. = not determined. Table adopted from Scholze et al. 2020.

6.3 Experimental assessment of QIVIVE predicted *in vivo* AGD effects

6.3.1 Fludioxonil, cyprodinil and dimethomorph

The ability of fludioxonil, dimethomorph, and cyprodinil to induce shortened AGDs after gestational exposure was investigated in a reproductive/developmental toxicity screening study in the rat (modified OECD TG 421)(OECD 2016a). Each pesticide was tested with at least three doses, selected to fall in the range at which QIVIVE predicted shortened AGDs in males (Figure 7C, "predicted window for *in vivo* activity"). At low doses we anticipated only a weak *in vivo* response (close to the sensitivity limit), while at higher doses, we expected a clear response in terms of altered AGDs, without any interfering maternal toxicity. The high doses were chosen below the threshold for maternal toxicity observed in other studies.

The observed dose-response pattern for AGD reductions in the newborn male pups were not as clear-cut as we had expected, but all three pesticides did produce statistically significant AGD reductions towards the centre of the predicted dose range (Figure 7C). Cyprodinil, at 60 mg/kg/day, produced statistically significant AGD shortening, but not at 180 mg/kg/day; this dose has been shown to induce maternal liver toxicity (EFSA, 2005). Fludioxonil, at 180 mg/kg/day, led to AGD changes similar in magnitude to those observed at 60 mg/kg/day, but with high variations between litters, which may have prevented the values from reaching statistical significance. In the first of the dimethomorph studies, the two lowest doses led to statistically significantly different AGDs, but not at the higher dose of 60 mg/kg/day (Figure 7C, red symbols). This motivated us to repeat in a second study the same doses together with a high dose but with a different rat strain. In this study, statistically significantly shortened AGD were seen at doses of 60 and 180 mg/kg/day (Figure 7C, black symbols). At 180 mg/kg/day we observed indications for a mild maternal toxicity (increased post-implantation and perinatal loss, higher postnatal death rate, Appendix 3). There were no statistically significant effects on nipple retention at PD14 in the studies with fludioxonil, cyprodinil or dimethomorph (Appendix 3), but in the second dimethomorph study retained nipples were seen at doses of 7 and 180 mg/kg/day (Appendix 3).

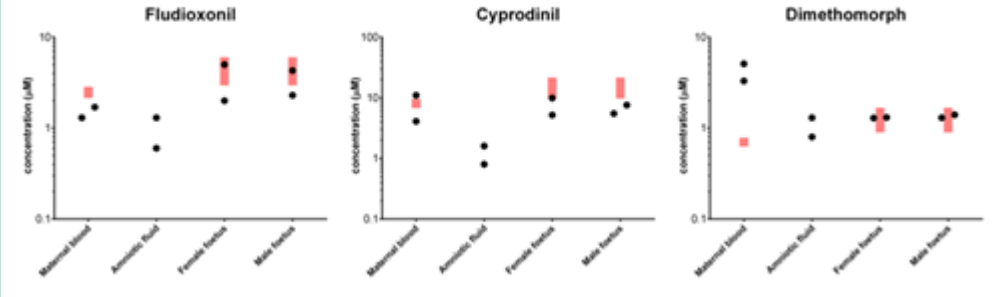


FIGURE 6. Measured and physiologically-based kinetic (PBK)-model-simulated pesticide concentrations in maternal and fetal plasma as well as amniotic fluid after repeated dosing of fludioxonil (60mg/kg/bw), cyprodinil (60mg/kg/bw), and dimethomorph (20mg/kg/bw) (GD7 - GD21). The ranges of simulated concentrations are shown as red bars and refer to average plasma concentrations on gestational day (GD) 21 (dams) or fetal concentrations on GD15 - GD18. Measurements are shown as black dots representing one dam (maternal blood), one litter (amniotic fluid) or gender-specific litter means (fetus) and were taken on GD21 approximately 90 min after dosing by oral gavage. Figure modified from Scholze et al. 2020.

The predicted levels of fludioxonil, cyprodinil and dimethomorph in the fetal compartment and in the dams' plasma were evaluated by chemical analysis of fetal samples harvested by Caesarean section between GD15-18, and of maternal blood samples drawn on GD21, respectively. The measured concentrations in the fetuses and in the dams' plasma agreed well with the PBK simulations (Figure 6). The ratios between observation and prediction were in the range between 0.5 and 2, showing good accuracy of the PBK model. However, the maternal plasma levels predicted for dimethomorph were approximately 10 times lower than the analytically determined concentrations.

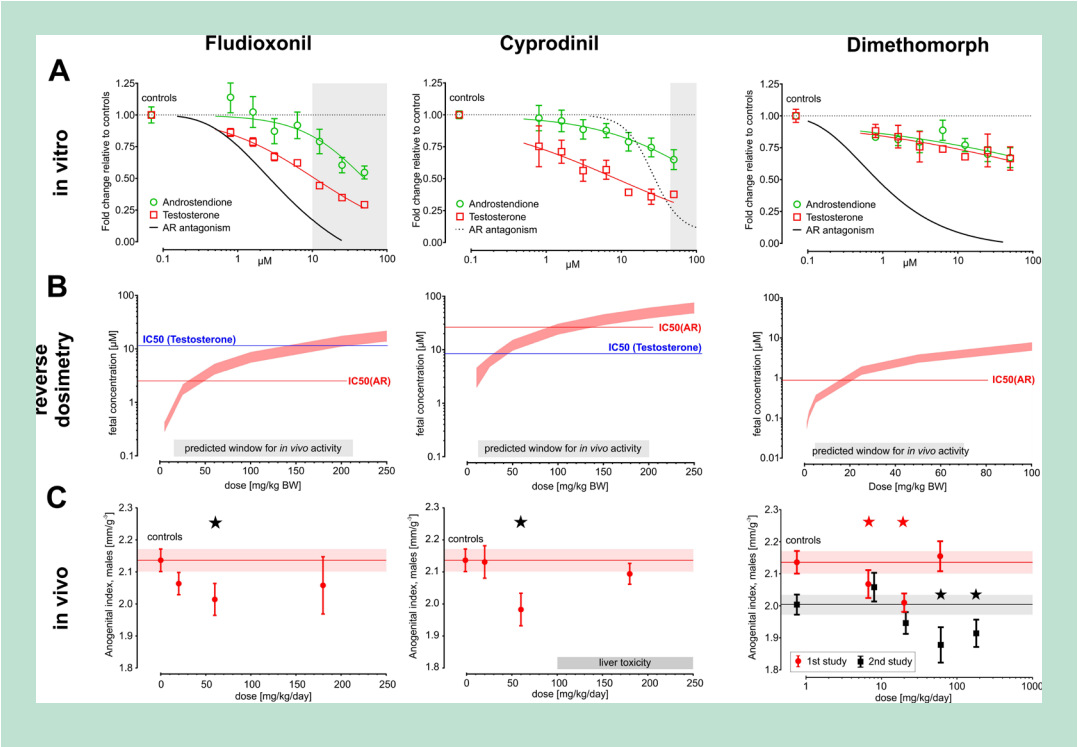


FIGURE 7. Assessment of the utility of the QIVIVE for anticipating shortened AGDs in male offspring following gestational exposure to fludioxonil, cyprodinil, and dimethomorph. A) Concentration-response curves for AR antagonism, testosterone and androstenedione inhibition *in vitro*. Data represent mean ± SEM (n = 3). Grey areas indicate cytotoxic concentration ranges in the H295R assay. B) Fetal levels of the pesticides predicted by PBK modelling as a function of the *in vivo* dose. The horizontal lines illustrate the *in vitro* activities. The horizontal grey areas ("predicted window for *in vivo* activity") depict the dose ranges at which a shorten AGD cannot be ruled out. The red shadings reflect different kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see Materials and Methods). C) Anogenital distance measured at birth following exposure from gestational day (GD)7 to GD21 to fludioxonil, cyprodinil (20, 60 and 180 mg/kg bw/day) or dimethomorph (6.7, 20 or 60 mg/kg bw/day) (1st sudy on Wistar rats); A follow-up study was conducted on Sprague Dawley rats (6.7, 20, 60 or 180 mg/kg bw/day) for dimethomorph. The grey bar for cyprodinil illustrates liver hypertrophy reported *in vivo* in rodent studies (EFSA Draft Assessment Report). Data represents mean ± 95% confidence belt (n = 6-10 litters). * $p<0.05$ for comparison to the control group (multiple contrast testing in mixed effect ANOVA model). Figure taken from Scholze et al. 2020.

6.3.2 Imazalil

Imazalil has not previously been properly investigated for its endocrine disrupting potential *in vivo*. A range finding study showed that all three tested doses (40, 55 and 70 mg/kg bw/day), caused maternal toxicity and disrupted parturition. Therefore, a dose of 24 mg/kg bw/day was chosen as the highest dose to be investigated in the main developmental study for dams to deliver. Additionally, a group receiving 72 mg/kg bw /day was included as part of the GD21 cohort.

In the definite study, AGD was measured on GD21 and on PD1 but no indications of decreased male AGD were seen (Figure 8). Neither effects on nipple retention were observed. More details on the study can be found in Appendix 3.4.

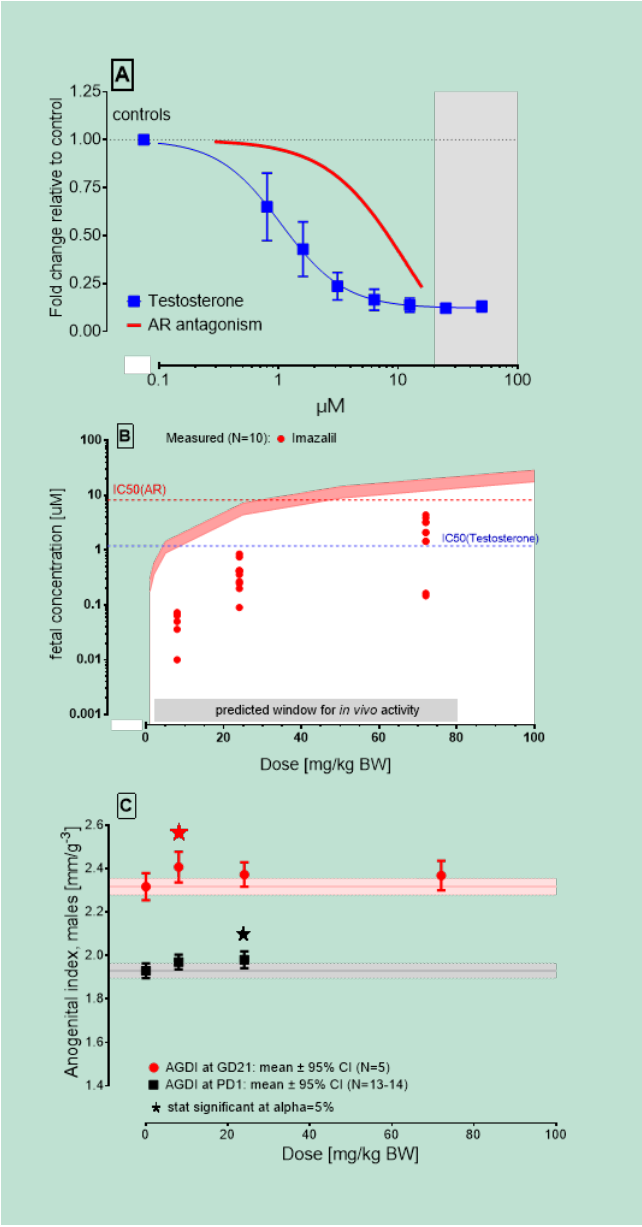


FIGURE 8. Assessment of the utility of the QIVIVE for predicting shortened AGDs in male offspring following gestational exposure to imazalil. A) Concentration-response curves for AR antagonism and testosterone inhibition *in vitro*. Data represent mean ± SEM (n = 3). Grey areas indicate cytotoxic concentration ranges in the H295R assay. B) Fetal levels of imazalil modeled as a function of the *in vivo* dose. The horizontal lines illustrate the *in vitro* activities. The horizontal grey areas ("predicted window for *in vivo* activity") depict the dose ranges at which a shorten AGD cannot be ruled out. The red shadings reflect different kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see Materials and Methods). Fetal levels at (GD21) are shown for male-specific litter means. C) Anogenital distance measured at GD21 and PD1 following exposure from gestational day (GD)7 to GD21 to imazalil (8, 24 and 72 mg/kg/day) in Sprague Dawley rats. Data represents mean ± 95% confidence belt. * $p<0.05$ for comparison to the control group (multiple contrast testing in mixed effect ANOVA model).

The internal exposure levels of imazalil in dams and fetuses are shown in figure 9. In all compartments a clear dose-related trend at increasing doses were present, however, the data variation was higher than observed in the studies on fludioxonil, cyprodinil or dimethomorph. Gender-specific differences in the fetal levels could not be confirmed, the fetal plasma levels were

slighty higher than the maternal plasma levels, and the imazalil concentrations in amniotic fluid were significantly lower than those measured in the fetal compartment.
The simulated fetal concentrations clearly overestimated the measured levels in the pups (Figure 8B), e.g. at 24 mg/kg/day by a factor of 20 (Table 3). This suggests that the compound was either less absorbed than we expected or quicker metabolized or eliminated than assumed by the PBK model. We rule out a dramatic overestimation of the absorption rate as this would be in clear disagreement to the reported kinetic data from the DARs, but we speculate that the lack of data support for a reliable estimation of the elimination might have led to this clear underestimation of the elimination parameters. Nevertheless, the internal exposure levels are far too low to reach concentration ranges that would provoke an *in vitro* activity (Figure 8C), suggesting that the lack of sufficient high imazalil concentrations in the fetus was most likely responsible for the negative AGD findings in the pup.

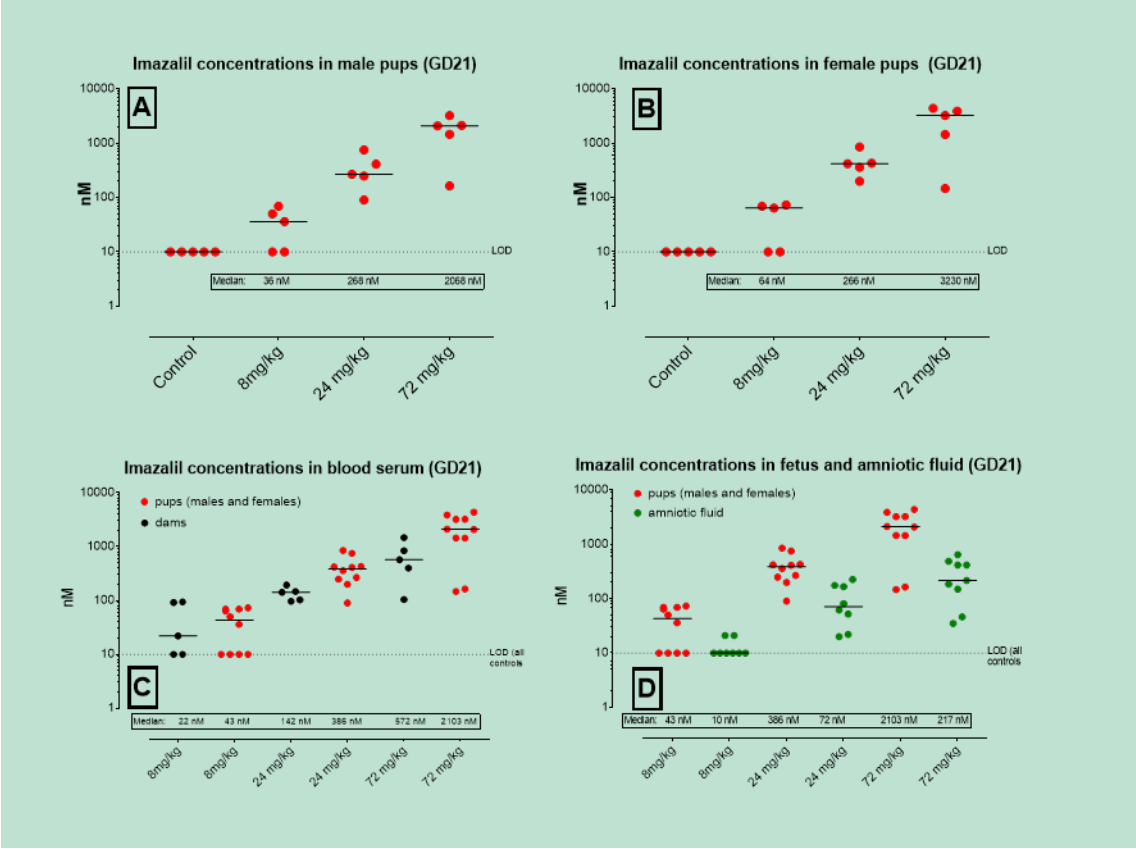


FIGURE 9. Measured concentrations in maternal and fetal plasma as well as amniotic fluid after repeated dosing of imazalil (8, 24 and 72 mg/kg/day) (GD7 - GD21). Measurements are shown as black dots representing one dam (maternal blood), one litter (green dots for amniotic fluid) or gender-specific litter means (red dots for pups) and were taken on GD21 approximately 90 min after dosing by oral gavage. The median is shown as horizontal line, with values listed below the data scatter. A) and B) show the concentrations in male and female pups, respectively, C) the fetal levels of both genders against the maternal concentrations, and D) the fetal levels of both genders against amniotic fluid.

The EU Research project EUROMIX (Grant Agreement n°633172) recently developed a generic PBK model for adult rats where imazalil was extensively studied by kinetic *in vitro* assays and in silico tools. From the imazalil-specific model parameters estimated by EUROMIX we found three kinetic parameters compatible with our PBK model structure, namely the renal excretion rate (estimated as 0.09 L/h), the metabolic clearance (estimated as 6 L/h) and the intake dose absorbed by the gut (80%) (https://zenodo.org/record/3472609#.X4bS6TI7mUk). These data were not available to us (in DAR) at the beginning of the JANUS project, and PBK simulations

were rerun with the EUROMIX model parameter after the *in vivo* study was conducted. To use the fixed value of 80% absorption, the intake dose was reduced by 80% and the fecal excretion rate (K_{Feces}) was fixed to zero. The change of the metabolic clearance rate to a much higher value resulted into significantly lower fetal concentrations by the PBK simulations, and agreed well with the imazalil levels measured at GD21 after repeated dosing of 8, 24 and 72 mg/kg/day (Figure 10). Moreover, the PBK model-simulations suggest fetal concentrations at these doses that are well below *in vitro* concentrations at which a relevant inhibition of testosterone synthesis or AR antagonisms was detected, and according to the QIVIVE, we should not have expected a reduced AGD in male offspring at the investigated dose ranges.

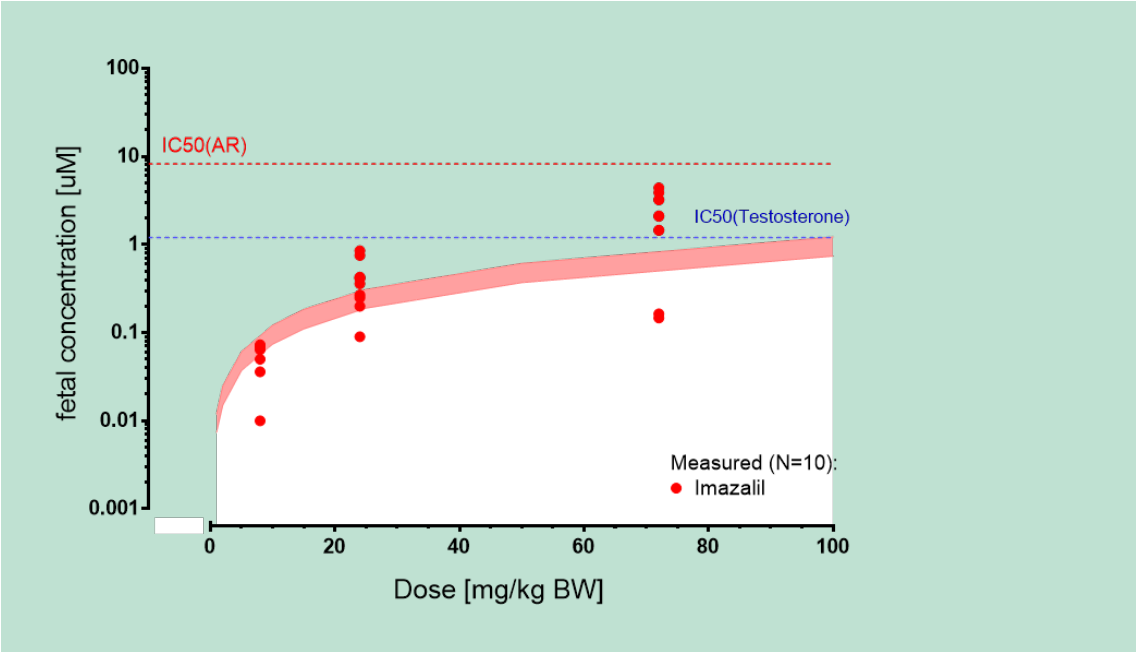


FIGURE 10. Fetal levels of imazalil predicted by PBK modelling on basis of EUROMIX model parameters as a function of the *in vivo* dose. The horizontal lines illustrate the *in vitro* activities. The red shadings reflect different kinetic model assumptions for describing the exposure transport between placenta and fetal compartment (see Materials and Methods). Fetal levels at (GD21) are shown for sex-specific litter means.

6.4 Placental transfer of pesticides

We used measured levels of the test compounds in amniotic fluid and blood from dams and fetuses to calculate an estimated total placental transfer (ETPT), as shown in Table 4.
For procymidone (40mg/kg), finasteride (1 mg/kg), fludioxonil (60mg/kg) and cyprodinil (60mg/kg), for which the transfer calculations were made from single exposure dose groups, the ETPT was between 2-9% (Table 4). For vinclozolin only the metabolites (M1 and M2) and not the parent compound could be detected in the dams and fetal blood. This might be due to the fact that vinclozolin in solution is rapidly hydrolyzed to its major metabolites M1 and M2. In general vinclozolin (40mg/kg) showed higher ETPTs than for the previously mentioned compounds and there seems to be a slightly higher transfer of M2 (12-23%) compared to M1 (8-20%).
For imazalil a dose-dependent ETPT was observed at 2-22% with the highest transfer seen at the highest dose of 72 mg/kg. In contrast, for dimethomorph (7 & 20mg/kg) the ETPT was between 1-3% and here the transfer did not seem to be dose-dependent.

Finally, for linuron there was a broader span or variation between the ETPT (2-39%) than seen for the other compounds, when looking at the metabolites. The highest transfer was seen for the M2 metabolite, but overall the transfer was not affected by increasing doses (Table 4).

TABLE 4. Estimated total placental transfers (%) of various pesticides and some metabolites determined in rats at gestational day 21

Treatment	Pesticide levels (nmol): Parent compound				ETPT (%)
	Dam no.	Dams plasma	Males plasma	Females plasma	Amniotic fluid	
Imazalil 8mg/kg	11	0,36	0,01	0,02	0,00	6,90
	15	1,94	0,02	0,02	0,00	1,80
	64	0,00	0,00	0,00	0,00	0,00
	65	0,00	0,00	0,00	0,00	0,00
	66	1,91	0,02	0,02	0,01	2,55
Imazalil 24mg/kg	26	2,64	0,08	0,11	0,03	8,33
	27	3,04	0,07	0,11	0,02	6,66
	28	3,71	0,17	0,18	0,09	11,84
	77	1,99	0,12	0,09	0,02	11,65
	78	2,11	0,03	0,06	0,01	4,35
Imazalil 72mg/kg	30	28,19	0,83	1,07	0,17	7,31
	31	15,96	0,61	0,92	0,06	9,95
	32	7,78	0,44	1,07	0,17	21,52
	79	1,95	0,05	0,05	0,02	6,11
	80	9,19	0,59	0,40	0,07	11,62
Procymidone 40mg/kg	11	97,09	3,95	0,00	0,75	4,84
	12	127,51	2,32	2,46	0,78	4,37
	13	178,55	1,77	3,96	1,67	4,14
	14	202,36	1,23	5,16	1,38	3,84
	15	128,52	2,40	2,84	0,96	4,83
	18	23,43	0,79	1,00	0,33	9,03
Finasteride 1mg/kg	9	1,37	0,01	0,01	0,00	1,40
	10	1,84	0,02	0,01	0,00	1,94
	11	0,79	0,01	0,01	0,00	2,56
	12	1,46	0,02	0,01	0,01	2,65
Fludioxonil 60mg/kg	81	26,62	0,58	0,48	0,24	4,87
	82	34,27	1,05	1,18	0,52	8,02
Cyprodinil 60mg/kg	91	212,61	1,65	2,28	0,64	2,15
	92	82,92	1,27	1,21	0,64	3,76
Dimethomorph 20mg/kg	104	114,24	0,32	0,27	0,32	0,80
	105	61,67	0,47	0,45	1,08	3,25
Dimethomorph 6.7mg/kg	27	2,69	0,02	0,01	0,02	1,93
	28	12,74	0,10	0,09	0,04	1,84
	60	10,45	0,00	0,02	0,04	0,65
	61	14,87	0,07	0,07	0,04	1,22
	82	2,55	0,01	0,01	0,02	1,70

Treatment	Pesticide levels (nmol): Parent compound and Metabolites													ETPT (%)		
	Dam no.	Dams plasma			Males plasma			Females plasma			Amniotic fluid					
		Parent	M1	M2	Parent	M1	M2	Parent	M1	M2	Parent	M1	M2	Parent	M1	M2
Vinclozolin 40mg/kg	21		45,52	8,99		3,08	0,58		2,67	0,97		3,14	0,18		19,55	19,31
	22		91,70	15,69		6,17	1,02		4,13	1,64		3,46	0,30		15,01	18,87
	24		55,49	7,43		2,76	0,51		3,29	1,02		3,44	0,20		17,09	23,28
	25		158,31	24,32		6,03	1,53		4,10	2,53		3,83	0,29		8,82	17,87
	26		75,15	17,53		5,30	0,92		3,08	1,68		2,75	0,20		14,80	15,97
	28		130,07	19,87		4,11	0,71		3,11	1,56		3,56	0,28		8,29	12,87

Linuron 25mg/kg	21	17,91	17,99	25,21	0,48	1,48	4,10	0,47	1,41	3,73	0,22	0,50	2,00	6,54	18,83	38,96
	23	33,02	61,87	64,54	0,58	2,08	3,07	0,36	1,11	3,05	0,09	0,37	0,51	3,15	5,76	10,28
	24	38,33	44,75	75,88	0,35	1,15	2,37	0,23	0,64	2,24	0,07	0,15	0,48	1,69	4,34	6,70
Linuron 50mg/kg	25	77,74	126,53	229,09	1,47	3,29	8,26	0,74	1,91	4,68	0,44	1,12	2,82	3,40	5,00	6,88
	27	30,91	45,81	175,74	0,81	2,24	7,72	0,76	2,26	7,88	0,41	1,05	5,13	6,40	12,09	11,79
	28	46,63	101,15	221,17	1,10	2,99	7,72	0,79	2,10	5,49	0,42	1,37	3,55	4,97	6,38	7,58
Linuron 50mg/kg	29	99,66	291,26	438,10	1,03	5,61	8,68	0,59	3,43	5,43	0,70	3,57	5,39	2,33	4,33	4,45
	31	73,59	294,97	454,60	0,89	5,81	10,42	0,92	6,15	10,57	0,14	0,93	2,34	2,66	4,37	5,13
	32	72,62	143,65	296,24	2,40	7,93	15,09	0,98	3,68	7,24	0,29	1,26	2,74	5,06	8,96	8,46

6.5 Incorporation of *in vitro* metabolism

Pesticides were incubated with S9 and the resulting metabolite soup was added to the *in vitro* assays. Following this procedure, the AR antagonistic potencies decreased dramatically, which is reflected by increasing IC50 values for five compounds; one exception being procymidone (Table 5). This suggests that the parent compound was mainly responsible for the observed AR antagonists and not metabolites.

Table 5: The IC_{50} from the AR antagonist assay without and with metabolism

Parent Compound	IC_{50} (µM)	IC_{50} (µM) after metabolism
Prochloraz*	3	10
Fludioxonil*	0.7	15
Imazalil*	3	10
Vinclozolin*	0.2	21
Procymidone*	0.2	0.1

IC50: The concentration of pesticide that is able to inhibit activation of the human androgen receptor (activated by 0.1 nM R1881) This table is modified from Taxvig, 2020.

For prochloraz, imazalil, dimethomorph and linuron we found no differences in effects between the compounds tested alone or with S9 in the H295R assay.
For fludioxonil and cyprodinil the decrease in testosterone and androstenedione seen for the parent compound, was not seen following incubation with S9.
In contrast, for fludioxonil, cyprodinil procymidone and vinclozolin the significant increase in estradiol seen with the parent compound was even more pronounced with S9. This more pronounced increase in estradiol levels following incubation with S9 might suggest that some of the metabolites of these compounds are able to affect estradiol production more potently than the parent compounds.

7. Discussion

We developed a QIVIVE approach for anti-androgenicity that predicts concentrations of a test compound in the fetal compartment attained after maternal dosing during the “male programming window”. Comparison of simulated fetal levels with *in vitro* active concentration ranges for AR antagonism and suppression of androgen synthesis enabled us to anticipate whether *in vivo* effects on AGD changes should occur. We demonstrated the applicability of the QIVIVE approach to three well-studied anti-androgenic compounds (vinclozolin, linuron and procymidone). Extension of this approach to nine current-use pesticides, all with *in vitro* evidence for anti-androgenicity but missing *in vivo* data, produced seven predicted *in vivo* actives. We confirmed our predictions for three of these seven pesticides (cyprodinil, fludioxonil and dimethomorph) relatively accurately (within a factor of 5). The internal exposures *in vivo* for imazalil were initially hard to predict, but new available kinetic data made it possible to develop a better PBK model. This new PBK model was able to predict the actual fetal imazalil levels with reasonable accuracy. For two of the nine pesticides, λ-cyhalothrin and pyrimethanil, the simulated fetal concentrations were below the *in vitro* active concentrations and are therefore expected not to cause any effects on AGD *in vivo.*

Our method rests on the assumption that the *in vitro* anti-androgenic activity of a chemical (AR antagonism or inhibition of androgen synthesis) will lead to a shortened AGD in male offspring, as long as the kinetics of the substance permit that *in vitro* active concentrations can be reached in the fetal compartment after maternal dosing below the toxic range. We further assume that there are no modulating factors that counteract the effects by changing endogenous hormone levels. Both kinetic and toxicodynamic considerations are therefore decisive in predicting whether an *in vivo* effect is likely to occur.

However, the converse reasoning is not admissible: If QIVIVE suggests that critical fetal concentrations cannot be reached after maternal dosing in the sub-toxic range, as was the case with λ-cyhalothrin and pyrimethanil (Figure 5), it is not possible to rule out *in vivo* activity. Other, as yet unknown, modes of action not captured by the *in vitro* assays might also lead to short AGDs (see discussion below). Thus, the accuracy of our QIVIVE is not only conditional on kinetics, but also on the selected *in vitro* effect profile. Both these assumptions require critical examination.

7.1 Toxicokinetics – uncertainties of the PBK model

Our PBK model was designed to be compatible with the data on absorption, distribution, metabolism, excretion (ADME) available from EU pesticide Draft Assessment Reports (DAR). We used these data to estimate compound-specific first-order elimination rates due to metabolism, renal clearance and excretion, without the need for predictive in silico tools or additional *in vitro* experiments. However, these elimination rates are based on kinetic measurements in non-pregnant animals reported in DARs. It is conceivable that these kinetic parameters are different during gestation, but data to further investigate this possibility were not accessible to us. Our modelling strategy was therefore to capture the kinetics of internal exposures during gestation as much as possible by employing physiological parameters as they change during gestation (e.g. organ weights, blood flow rates, etc.).

Unfortunately, there is no agreed schema by which ADME data for pesticides are measured and reported. There are great variations in terms of the physiological compartments sampled, the timing of sampling, the number of studies conducted and the ways in which kinetic measurements are documented (e.g. measurements for individual animals versus summarizing kinetic descriptors such as AUC). It was therefore not possible to pursue a uniform approach when deciding which data to use for deriving PBK model parameters. Rather, this had to be

done on a case-by-case basis. As a result, we occasionally obtained more than one set of model parameters that equally well described the concentrations reported in blood, urine and feces. Inevitably, therefore, some of the kinetic parameters we selected may not represent the correct elimination rates.
These limitations apply particularly to extensively metabolized substances with complex elimination patterns such as imazalil, where 24 hours after dosing at least 25 metabolites were discovered in urine and feces. Imazalil undergoes many different routes of metabolism, including epoxidation, epoxide hydration, oxidative O-dealkylation, imidazole oxidation and scission, and oxidative N-dealkylation (DAR Imazalil Annex B.5). For compounds with such complex and rapid metabolism, analytical determinations of metabolite levels soon after dosing are needed, but in the case of imazalil such data were not available for time points earlier than 12 hours after dosing (Mannens et al., 1993). As demonstrated, imazalil was eliminated much faster than assumed in our first PBK simulations which lead to predicted fetal levels that were around 20-fold higher than the actual measured values in this study. Thus, *in vivo* kinetic data from the DAR reports provided not the sufficient details which were required for the estimation of the kinetic parameters, and an ADME *in vivo* study with a focus on establishing metabolite levels within the first 3-6 hours after last dosing available at the project start could have removed the uncertainties in our estimates. This led to a false positive prediction of *in vivo* activity regarding shortened AGDs. If the model parameters were replaced with more accurate estimates which were recently made public available by the EU research project EUROMIX (https://zenodo.org/record/3472609#.X4bS6Tl7mUk) and which were estimated from *in vitro* and in silico methods, the simulation of the PBK model resulted into fetal levels that were similar to those measured in the fetus at GD21. This model refinement demonstrates the importance of integrating all available *in vitro* and in silico approaches to support the estimation of kinetic model parameters, even in case of a simple PBK model structure. However, it also shows the limitation of kinetic *in vivo* studies that do not provide a sufficient detail of time-course data which are necessary to estimate highly dynamic kinetic processes for the PBK simulations, even when the kinetic *in vivo* studies were conducted on the same species and a comparable dose regime.

Our PBK model also assumed that the distribution of compounds between compartments, especially the bi-directional transport between placenta and fetus, is by passive diffusion, rather than active transport. Due to a lack of more detailed data, the fetus was modeled as a simple one-compartment, without consideration of further distribution processes within the compartment, compartment-specific protein binding or elimination processes. Due to these simplifications, it was not feasible to characterize the fetal compartment in terms of maximum plasma concentration Cmax or time to maximum plasma concentration Tmax. The only reliable estimate of the fetal internal exposure load was the AUC (area under plasma concentration-time curve) over the period of interest (GD15-GD17). Accordingly, we used AUC per hour as an approximation of average fetal concentrations.
Sensitivity analyses were performed (Appendix 1.5) and revealed the bi-directional transport of compounds between the placenta and the fetus as the most uncertain and sensitive element of our model simulations. Due to a lack of adequate data from in silico or *in vitro* methods, our only option of overcoming this uncertainty was by using a range of transplacental rates for the PBK simulations, which we considered equally possible, with a maximum relative difference between high and low clearance rates set as ±20%. Whether this range reflects a worst-case is debatable, but to our knowledge, no gestational PBK model for rats has been reported that suggests larger parameter differences, at least not for chemicals that have kinetic properties like those of the modeled pesticides.
Despite all these uncertainties, the simulations agreed rather well for six out of seven compounds with the measured values and did not, except for imazalil, deviate by more than a factor of five and often less than that. It appears that our relatively simple PBK model structure, combined with the kinetic data contained in DARs was sufficient to approximate fetal concentrations reasonably well.

We used the measured pesticide levels in dams and foetuses to estimate the placental transfer. As we have only got analytical data for one time point i.e. GD21, we cannot estimate a transfer rate from these numbers. We would need measurements from at least two different time points to get a rough estimate of the rates, as we cannot anticipate that a steady-state situation has occurred even after 21 days of exposure of the dams. Thus, our result indicate the transfer at exactly this time point but this may be different if samples were taken at another point in time.

7.2 Toxicodynamics – from AR antagonism to AGD changes

Three of the pesticides selected for testing our QIVIVE predictions induced shortened AGDs, but notable are some unusually shaped dose-response curves. In each case, specific reasons for the observed response patterns may be conjured up. The lack of response at the highest dose of cyprodinil may be due to interference with maternal liver function (EFSA 2005). Furthermore, cyprodinil is an *in vitro* AhR activator and induces expression of CYP1A1 (Fang et al. 2013) which may have led to increased hepatic metabolism of steroids at the high dose, thereby preventing the induction of AGD changes. As discussed above, such phenomena cannot be captured by our PBK modelling. With fludioxonil, there were unusually high variations in AGD at the highest dose, which have prevented the AGD values from reaching statistical significance. The non-monotonic dose-response pattern seen with dimethomorph was not replicated in a second study with larger numbers of animals, but a different rat strain. This requires further investigation. In any case, however, both studies produced evidence of AGD changes. The example of dimethomorph highlights that our QIVIVE model may not be capable of predicting dose-response patterns, rather it can help establishing whether compounds induce AGD changes in a qualitative sense. It has to be kept in mind that the *in vivo* AGD data for dimethomorph were not easily reproduced from experiment to experiment and therefore it is not easy to fix the response level which an alternative model should aim at predicting.
Furthermore, there are differences in the effect strength with which AR antagonists produce AGD changes, pointing to complex relationships between diminished androgen action in the fetus and AGD changes. The sex-specific differentiation of the perineum, the area between anus and genitalia, is dependent on the stimulation of the growth of the perineal muscles levator ani and bulbocavernosus complex (LABC) which is mediated by AR activation. Certain AR antagonists such as vinclozolin, flutamide and procymidone can produce severely shortened AGDs that approach female control values, while most other chemicals, including some phthalates, butylparaben or bisphenol A induce much less marked shortenings (Schwartz et al. 2019). Clearly, cyprodinil, fludioxonil and dimethomorph fall into the latter category with changes in AGD of around 15% when related to the AGD of female offspring (Table 3). The reasons for these differences in effect strength are unknown, but an appealing possibility discussed by Schwartz et al. (2019) is the estrogenicity of some of the anti-androgens.
In any case, our QIVIVE is not designed to predict the effect strength of AGD changes, nor the shapes of dose-response curves. As developed and presented here, the approach is only capable of anticipating *in vivo* activity in a qualitative sense, as a binary “active” – “inactive” option.
The complex relationships between receptor-mediated and cellular responses and AGD changes argue for inclusion of as many *in vitro* anti-androgenicity read-outs as possible, at a minimum AR antagonism and suppression of androgen synthesis, as in this project. With certain compounds, there may be merit in additionally including measures of other anti-androgen-related mechanisms of action (e.g. 5α-reductase inhibition or blocking of the membrane androgen receptor) as well as *in vitro* estrogenicity.
Initially we considered to include prostaglandin D2 synthesis inhibition *in vitro* in our approach as a potential relevant antiandrogenic mechanism of action due to the hypothesis put forward by Kugathas et al. (2016). Therefore a comparison of the potencies on prostaglandin synthesis inhibition of eight of the included pesticides in this project with known *in vivo* effects on AGD

was performed (Appendix 2.3). We concluded that we could not derive any support for the involvement of this mechanism of action in the antiandrogenic effect based on the present limited data and that more data are needed in order to reach any conclusion on this.

7.3 Prediction of a wider anti-androgenic effect spectrum

The chemicals capable of producing severely feminized AGDs typically produce an anti-androgenic effect spectrum also comprising of retained nipples, prostate agenesis, penile malformations, and reduced weights of androgen-dependent organs (reviewed in Schwartz et al. 2019). However, with agents that produce milder shortened AGDs, these effects are not consistently observed, indicating that there is no clear relationship between the various manifestations of anti-androgenicity. This is the case with fludioxonil, cyprodinil and dimethomorph at the doses tested here. With the exception of dimethomorph, these chemicals did not induce nipple retention. Thus, it is difficult to anticipate additional *in vivo* anti-androgenic effects, even when AGD changes are predicted. However, this is an issue related to our incomplete understanding of the processes leading to the various physical manifestations of androgen insufficiency, rather than a weakness of our QIVIVE approach. This calls for further studies of the diversity of patterns/profiles of anti-androgenicity.

7.4 False negative and false positive predictions

False negatives
The complex processes leading to shortened AGDs in male pups may not only be induced by anti-androgens. Mild analgesics such as paracetamol or acetylsalicylic acid can also induce shortened AGDs, although these substances are not known AR antagonists or suppressors of androgen synthesis (Kristensen et al. 2011; Schwartz et al. 2019). The mechanisms by which changes in AGD arise after exposure to analgesics remain to be elucidated and compounds that act by such unknown mechanisms will not be detected with our present approach.
False negative predictions can also occur with substances where the levels in the fetal compartment predicted to be attainable after maternal dosing do not reach *in vitro* active AR antagonistic or androgen suppressing effective concentrations. For instance, despite negative predictions of AGD changes predicted for λ-cyhalothrin and pyrimethanil, we cannot rule out that such substances may lead to incomplete masculinization of male AGDs by mechanisms independent of diminished androgen action, as discussed above.

False positives
In some cases, the *in vitro* active concentration ranges of test compounds are lower than the concentration ranges predicted as attainable in the fetal compartment *in vivo*, in other words: all simulated fetal levels are anticipated to be active. False positive predictions might arise if there are bioavailability differences between the *in vitro* and *in vivo* situation. Protein binding can be a crucial factor leading to higher *in vitro* effective free concentrations than *in vivo*, because smaller proportions of compounds are bound to proteins, due to lower *in vitro* protein levels. In principle, the issue could be addressed by simulating the free, unbound fractions of parent compounds and metabolites, but this will require the availability of relevant data for the estimation of model parameters describing the kinetics in the fetus, which may pose difficulties. It is at present hard to judge whether the additional data requirements that this entails will lead to significant improvements of the predictive power of our QIVIVE.
False positives could also arise if high doses cause changes in metabolism of compounds to an inactive metabolite. Another possibility for false positive predictions might result from overestimations of internal maternal exposure levels due to a lack of sufficient toxicokinetic data, which was the case with the initial PBK model for imazalil.

7.5 Testing strategy

Several OECD test guidelines for reproductive and developmental toxicity require the measurement of AGD in offspring and/or fetuses (OECD TG 414, 421/422, 443), (OECD 2012, 2015, 2016a, 2018), and this endpoint is vital for the identification of endocrine disrupting chemicals. AGD changes can be used for estimating NOAELs or benchmark doses, which form the basis for establishing health-based guidance values (OECD 2013).
Our QIVIVE approach may prove to be a useful tool for the prioritization of chemicals for *in vivo* testing. It is applicable to chemicals assumed to act via a specific pathway, the induction of AGD changes through androgen insufficiency via AR antagonism or suppression of androgen synthesis. We argue that *in vitro* AR antagonists and androgen synthesis suppressing chemicals predicted to be active *in vivo* should be treated as if they were *in vivo* actives, even when appropriate *in vivo* data are not available. This stance should not preclude confirmatory testing, if the necessary resources can be made available. However, we suggest that rather than mobilizing resources for confirming predicted *in vivo* positives, chemicals with *in vitro* anti-androgenic activity, but anticipated to be inactive *in vivo* should be prioritized for *in vivo* testing. Such a strategy will be more resource-effective and ethical than focusing on predicted *in vivo* actives. To gain an impression of the extent of the testing need, we suggest to evaluate for all pesticides authorized for use in the EU i) whether ADME data contained in DAR are compatible with our QIVIVE approach and ii) whether relevant *in vitro* anti-androgenicity data are available. If both conditions are met, our approach can be utilized to identify likely *in vivo* active candidates and to make decisions about further testing.

8. Conclusions

For six chemicals (procymidone, vinclozolin, linuron, fludioxonil, cyprodinil, and dimethomorph) with *in vitro* antiandrogenic activity we have shown that our QIVIVE approach can successfully predict the fetal levels attained after maternal dosing, as well as their *in vivo* anti-androgenicity as shown by reductions of AGDs. In general the PBK models predicted the internal fetal exposure levels within 5-fold.

For the fourth pesticide, imazalil, only the final updated PBK model correctly estimated the internal exposure levels when kinetic model parameters were estimated from *in vitro* and *in silico* approaches. The fetal levels of imazalil attained at the tested *in vivo* doses were too low to be in the range of *in vitro* active concentrations, and thus according to the QIVIVE a reduced AGD in male pups was not expected.

Our QIVIVE approach is applicable to agents that produce androgen insufficiency by AR antagonism and suppression of androgen synthesis and has great potential for minimizing unnecessary *in vivo* testing.

9. Perspectives

Our QIVIVE approach is based on PBK models that mimic rat physiology, which will be of utility for *in vivo* test prioritization. Thus we recommend that our approach is applied to all approved pesticides in the EU for which an effect on AGD has not been measured so far. Our approach can also be used for other chemicals for which a certain amount of kinetic data is available either from *in silico*, *in vitro* or *in vivo* studies.

In the long run our vision is to develop the QIVIVE approach based on exclusively human PBK models in order to obtain more human-relevant predictions that eliminate the need for species-species extrapolations. If realized, such approaches might have an even greater impact on the reduction of the use of animals for chemical risk assessment.

Furthermore, data contained in this project can prove to be useful for development of quantitative 'Adverse Outcome Pathways' (AOP), e.g. for an AOP for effects on AGD caused by disturbed AR signaling.

Finally, new approach methodologies like this QIVIVE approach may prove to be useful in the future chemical risk assessment for regulatory purposes, especially when PBK models based on human physiology become available.

10. Literature

Christiansen S, Kortenkamp A, Axelstad M, Boberg J, Scholze M, Jacobsen PR, et al. 2012. Mixtures of endocrine disrupting contaminants modelled on human high end exposures: An exploratory study in rats. Int. J. Androl.; doi:10.1111/j.1365-2605.2011.01242.x.

Ding H, Zheng W, Han H, Hu X, Hu B, Wang F, et al. 2017. Reproductive toxicity of linuron following gestational exposure in rats and underlying mechanisms. Toxicol. Lett.; doi:10.1016/j.toxlet.2016.12.013.

EFSA (European Food Safety Authority). 2005. Cyprodinil. Available: http://dar.efsa.europa.eu/dar-web/provision/request/subid/41.

EFSA (European Food Safety Authority). 2015. Rapporteur Member State assessment reports submitted for the EU peer review of active substances used in plant protection products. Eur. Food Saf. Auth. Available: http://dar.efsa.europa.eu/dar-web/provision.

EFSA (European Food Safety Authority). 2019. https://www.efsa.europa.eu/en/consultations/consultationsclosed?title=active%20substance

Emond C, Birnbaum LS, DeVito MJ. 2006. Use of a physiologically based pharmacokinetic model for rats to study the influence of body fat mass and induction of CYP1A2 on the pharmacokinetics of TCDD. Environ. Health Perspect.; doi:10.1289/ehp.8805.

European Commission. 2014. Alternatives to animal testing and safety assessment of chemicals. Available: https://ec.europa.eu/jrc/en/research-topic/alternatives-animal-testing-and-safety-assessment-chemicals.

Fang C-C, Chen F-Y, Chen C-R, Liu C-C, Wong L-C, Liu Y-W, et al. 2013. Cyprodinil as an activator of aryl hydrocarbon receptor. Toxicology 304:32–40; doi:10.1016/J.TOX.2012.11.018.

Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, et al. 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. APMIS 109: S302–S318.

Gray LE, Wilson V, Noriega N, Lambright C, Furr J, Stoker TE, et al. 2004. Use of the laboratory rat as a model in endocrine disruptor screening and testing. ILAR J.; doi:10.1093/ilar.45.4.425.
Hartung T, Rovida C. 2009. Chemical regulators have overreached. Nature 460:1080–1081; doi:10.1038/4601080a.

Hass U, Scholze M, Christiansen S, Dalgaard M, Vinggaard AM, Axelstad M, et al. 2007. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. Environ. Health Perspect. 115:122–128; doi:10.1289/ehp.9360.

Hecker M, Hollert H, Cooper R, Vinggaard A, Akahori Y, Murphy M, et al. 2011. The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study. Environ. Sci. Pollut. Res. 18:503–515; doi:10.1007/s11356-010-0396-x.

Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. 2008. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. Curr Urol Rep 9: 137–142.

Juul A, Almstrup K, Andersson AM, Jensen TK, Jorgensen N, Main KM, et al. 2014. Possible fetal determinants of male infertility. Nat Rev Endocrinol 10:553–562; doi:10.1038/nrendo.2014.97.

Kavlock RJ, Bahadori T, Barton-Maclaren TS, Gwinn MR, Rasenberg M, Thomas RS. 2018. Accelerating the Pace of Chemical Risk Assessment. Chem. Res. Toxicol. 31:287–290; doi:10.1021/acs.chemrestox.7b00339.

Kristensen DM, Hass U, Lesné L, Lottrup G, Jacobsen PR, Desdoits-Lethimonier C, et al. 2011. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. Hum. Reprod. 26:235–244; doi:10.1093/humrep/deq323.

Subramaniam K, Audouze K, Ermler S, Orton F, Rosivatz E, Scholze M and Kortenkamp A. Effects of Common Pesticides on Prostaglandin D2 (PGD2) Inhibition in SC5 Mouse Sertoli Cells, Evidence of Binding at the COX2 Active Site, and Implications for Endocrine Disruption. Environ. Health Perspect. 124(4), 452-459, 2016.

Lee HB and Blaufox MD. 1985. Blood-volume in the rat. J. Nuclear Med. 26 (1), 72-76.

Mannens G, Huang ML, Meuldermans W, Hendrickx J, Woestenborghs R, Heykants J. 1993. Absorption, metabolism, and excretion of risperidone in humans. Drug Metabol. Dispos. 21 (6):1134-1141.

McIntyre BS, Barlow NJ, Foster PMD. 2002. Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. Toxicol. Sci. 65:62–70; doi:10.1093/toxsci/65.1.62.

McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PMD. 2000. Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat. Toxicol. Appl. Pharmacol.; doi:10.1006/taap.2000.8998.

Mendiola J, Stahlhut RW, Jorgensen N, Liu F, Swan SH. 2011. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. Env. Heal. Perspect 119:958–963; doi:10.1289/ehp.1103421.

Mnif W, Hassine AIH, Bouaziz A, Bartegi A, Thomas O, Roig B. 2011. Effect of endocrine disruptor pesticides: A review. Int. J. Environ. Res. Public Health 8:2265–2303; doi:10.3390/ijerph8062265.

O'Flaherty EJ, Scott W, Schreiner C, Beliles RP. 1992. A physiologically based kinetic model of rat and mouse gestation: Disposition of a weak acid. Toxicol. Appl. Pharmacol. 112:245–256; doi:10.1016/0041-008X(92)90194-W.

OECD (Organisation for Economic Co-operation and Development). 2012. 443: Extended One-Generation Reproductive Toxicity Study. OECD Guidel. Test. Chem. Sect. 1–25.

OECD (Organisation for Economic Co-operation and Development). 2013. Guidance document in support of the test guideline on the extended one generation reproductive toxicity study No. 151.

OECD (Organisation for Economic Co-operation and Development). 2015. Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test. Test No. 422 Comb. Repeated Dose Toxic. Study with Reprod. Toxic. Screen. Test 1–19; doi:10.1787/9789264242715-en.

OECD (Organisation for Economic Co-operation and Development). 2016a. Reproduction / Developmental Toxicity Screening Test. Guidel. Test. Chem. (no.421). Organ. Econ. Coop. Dev. Paris. 1–11.

OECD (Organisation for Economic Co-operation and Development). 2016b. Test No. 458: Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals.; doi:10.1787/9789264264366-en.

OECD (Organisation for Economic Co-operation and Development). 2018. Test No 414: Prenatal developmental toxicity study.

Orton F, Rosivatz E, Scholze M, Kortenkamp A. 2011. Widely Used Pesticides with Previously Unknown Endocrine Activity Revealed as *in vitro* Antiandrogens. Environ. Health Perspect. 119:794–800; doi:10.1289/ehp.1002895.

Poulin P, Theil F. 2002. Prediction of Pharmacokinetics Prior to *In vivo* Studies. 1. Mechanism-Based Prediction of Volume of Distribution. J. Pharm. Sci. 91:129–156; doi:10.1002/JPS.10005.

Rosenmai AK, Nielsen FK, Pedersen M, Hadrup N, Trier X, Christensen JH, et al. 2013. Fluorochemicals used in food packaging inhibit male sex hormone synthesis. Toxicol. Appl. Pharmacol. 266:132–142; doi:http://dx.doi.org/10.1016/j.taap.2012.10.022.

Rosenmai AK, Winge SB, Möller M, Lundqvist J, Wedebye EB, Nikolov NG, Lilith Johansson HK, Vinggaard AM. 2021. Organophosphate ester flame retardants have antiandrogenic potential and affect other endocrine related endpoints *in vitro* and in silico. Chemosphere 263: 127703.

Scholze M, Boedeker W, Faust M, Backhaus T, Altenburger R, Grimme LH. 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environ. Toxicol. Chem.; doi:10.1002/etc.5620200228.

Scholze M, Taxvig C , Kortenkamp A, Boberg J, Christiansen S, Svingen T, Lauschke K, Frandsen H, Ermler S, Hermann SS, Pedersen M, Lykkeberg AK, Axelstad M & Vinggaard AM. Quantitative *In vitro* to *In vivo* Extrapolation (QIVIVE) for predicting reduced anogenital distance produced by anti-androgenic pesticides in a rodent model for male reproductive disorders. Environ. Health Perspect., 128 (11): 117005, 2020.

Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, Svingen T. 2019. Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. Arch. Toxicol. 93:253–272; doi:10.1007/s00204-018-2350-5.

Skakkebaek NE, Rajpert-De Meyts E, Buck Louis GM, Toppari J, Andersson AM, Eisenberg ML, et al. 2016. Male Reproductive Disorders and Fertility Trends: Influences of Environment and Genetic Susceptibility. Physiol Rev 96:55–97; doi:10.1152/physrev.00017.2015.

Swan SH. 2006. Prenatal phthalate exposure and anogenital distance in male infants. Environ. Health Perspect. 114: A88-9.

Taxvig, 2020. Advancing the application of alternative test methods in chemical risk assessment by incorporating metabolism into *in vitro* assays (InVita). https://www2.mst.dk/Udgiv/publications/2020/09/978-87-7038-228-1.pdf

Thankamony A, Pasterski V, Ong KK, Acerini CL, Hughes IA. 2016. Anogenital distance as a marker of androgen exposure in humans. Andrology 4:616–625; doi:10.1111/andr.12156.

Vinggaard AM, Nellemann C, Dalgaard M, Jørgensen EB, Andersen HR. 2002. Antiandrogenic Effects *in vitro* and *in vivo* of the Fungicide Prochloraz. Toxicol. Sci. 69:344–353; doi:10.1093/toxsci/69.2.344.

Vinggaard AM, Niemelä J, Wedebye EB, Jensen GE. Screening of 397 chemicals and development of a QSAR model for androgen receptor antagonism of chemicals. Chem.Res.Toxicol. 21(4), 813-23, 2008.

Wlodek ME, Westcott KT, Ho PWM, Se A. 2000. Reduced fetal, placental, and amniotic fluid PTHrP in the growth-restricted spontaneously hypertensive rat. Am J Physiol Regulatory Integrative Comp Physiol 279, R31–R38.

Appendix 1. PBK modelling data and equations

Appendix 1.1 Physiological parameters in the PBK models (in function of Gestational day=GD)

Parameter	Symbol	Range at GD6-GD20	Equation[a)]
Weights [kg]			
Body weight at GD0	BW_GD0	0.197	[own historical data]
Body weight, non-pregnant dam	BW_NP	0.212-0.248	BW_GD0 + 0.002429 * GD [own data]
kidney, pregnant dam	BW_Kidney	0.0017-0.0023	0.008*(BW_DAM - N_Concepti * BW_Conceptus)
Well-perfused tissues, pregnant dam	BW_ WellP	0.011-0.014	0.092*(BW_DAM - N_Concepti * BW_Conceptus) - BW_Liver_P
Poorly-perfused tissues, pregnant dam	BW_ PoorP	0.18-0.24	0.83*(BW_DAM - N_Concepti * BW_Conceptus)
liver weight, non-pregnant dam	BW_Liver_NP	0.0082-0.0088	0.04 * BW_GD0 * (BW_NP /BW_GD0)^0.48
liver weight, pregnant dam	BW_Liver_P	0.0088-0.0128	If GD≤6 then BW_Liver_NP, else BW_Liver_NP * (1- 0.0255*(GD - 6)
uterus weight, non-pregnant dam	BW_Uterus_NP	0.0023-0.0025	0.0022 * (BW_NP /BW_GD0)^0.48
uterus weight, pregnant dam	BW_Uterus_P	0.0033-0.022	If GD≤3 then BW_Uterus_NP else BW_Uterus_NP * (1+0.077*(GD -3)^1.6)
mammary tissue, non-pregnant dam	BW_Mam_NP	0.0021-0.0026	0.01 * BW_GD0 * (BW_NP /BW_GD0)^1.2
mammary tissue, pregnant dam	BW_Mam_P	0.0056-0.0173	BW_Mam_NP * (1+0.27*GD)
Fat, non-pregnant dam	BW_Fat_NP	0.014-0.015	0.07 * BW_GD0 * (BW_NP /BW_GD0)^0.48
Fat, pregnant dam	BW_Fat_P	0.016-0.021	BW_FAT_NP * (1+0.0165*GD)
Yolk sac placenta per fetus	BW_YPla	0-0.000032	If GD≤6 then 0, else if 6<GD≤10 then 8*(GD - 6)/1000^2, else 32*exp(-0.23 * (GD - 10))/1000^2 for GD>10
Chorioallantic placenta per fetus	BW_CPla	0-0.0008	If GD≤10 then 0, else 40*(exp(0.28*(GD-10))-1)/1000^2 for GD>10
Fetus	BW_Fetus	0-0.0036	5.092E-09*EXP(0.6413*GD) [own data]
Single conceptus	BW_Conceptus	0-0.0044	BW_YPla + BW_CPla + BW_Fetus
Litter size	N_Concepti	10	

Body weight, pregnant dam	BW_DAM	0.218-0.335	BW_NP + N_Concepti * BW_Conceptus + (BW_Liver_P - BW_Liver_NP) + (BW_Uterus_P - BW_Uterus_NP) + (BW_Mam_P - BW_Mam_NP) + (BW_Fat_P - BW_Fat_NP)
Blood flow fractions			
liver, non-pregnant dam	Q_Liver_F	0.18	
Kidney, non-pregnant dam	Q_Kidney_F	0.12	
Fat, non-pregnant dam	Q_Fat_F	0.07	
Well-perfused tissues, non-pregnant dam	Q_WellP_F	0.45	
Poorly-perfused tissues, non-pregnant dam	Q_PoorP_F	0.18	
Blood flow rates [L/h/kg]			
Cardiac output at GD0, total [L/h]	Q_Cardiac_GD0	12.5	
Cardiac output, non-pregnant dam	Q_Cardiac_NP	3.95-4.46	Q_Cardiac_GD0 * BW_GD0^0.74 * (BW_NP/BW_GD0)^0.7
liver, non-pregnant dam	Q_Liver	0.71-0.79	Q_Liver_F * Q_Cardiac_NP
Kidney, non-pregnant dam	Q_Kidney	0.47-0.53	Q_Kidney_F * Q_Cardiac_NP
Fat, non-pregnant dam	Q_Fat	0.28-0.31	Q_Fat_F * Q_Cardiac_NP
Well-perfused tissues, non-pregnant dam	Q_WellP	1.78-1.99	Q_WellP_F * Q_Cardiac_NP
Poorly-perfused tissues, non-pregnant dam	Q_ PoorP	0.71-0.79	Q_PoorP_F * Q_Cardiac_NP
Yolk sac placenta	Q_ YPla	0-0.92	If (GD≤6) then 0, else if (GD≤10) then 0.55*(GD - 6) /24, else 2.2*exp(-0.23 * (GD - 10))/24
Chorioallantic placenta	Q_ CPla	0-0.6	If (GD≤12) then 0, else ((0.1207*(GD-12))^4.36)/24;
Cardiac output, pregnant dam	Q_Cardiac_P	3.95-4.91	N_Concepti * (Q_YPla + Q_CPla) + Q_Cardiac_NP

[a)] if not otherwise stated, equations are from O'Flaherty et al. (1992).

Appendix 1.2 Kinetic equations for the rat PBK gestational model

All kinetic equations for the rat PBK gestational model are listed below (with the notation of model parameters referring to Tables 1 and S2):

Maternal blood compartment

$$C_{Plasma}\,(mg/l) = (Q_{Fat}*CV_{Fat} + Q_{WellP}*CV_{WellP} + Q_{PoorP}*CV_{PoorP} + Q_{Kidney}*CV_{Kidney} + Q_{Liver}*CV_{Liver} + N_{Concepti} * (Q_{YPla} * CV_{YPla} + Q_{CPla} * CV_{CPla}))$$
$$/ Q\_Cardiac\_p \qquad [1]$$

with Q_X referring to the blood flow rates of compartment X and $N_{Concepti}$ to the litter size.

Tissue compartment (Fat, well-perfused, poorly-perfused)

$$\frac{dA_X}{dt}(mg/hour) = R_{plasma} * Q_X * \left(C_{Plasma} - CV_X\right) \qquad [2]$$

$$C_X(mg/l) = A_X/V_X \quad \text{[Concentration in tissue compartment]} \qquad [3]$$

$$CV_X(mg/l) = C_X/Kp_X \quad \text{[Concentration leaving compartment]} \qquad [4]$$

with subscript notations X for Fat, WellP and PoorP.

Gastro-Intestinal absorption and distribution to the Portal

$$\frac{dA_{Lumen}}{dt}(mg/hour) = -\frac{dA_{Fecus}}{dt} - \frac{dA_{Portal}}{dt} + \frac{dA_{Biliary}}{dt} + INTAKE \qquad [5]$$

$$\frac{dA_{Fecus}}{dt}(mg/hour) = K_{feces} * A_{Lumen} \quad \text{[fecal elimination]} \qquad [6]$$

$$\frac{dA_{Portal}}{dt}(mg/hour) = K_{AS} * A_{Lumen} \quad \text{[adsorption to the liver]} \qquad [7]$$

$$\frac{dA_{Biliary}}{dt}(mg/hour) = K_{Biliary} * CV_{liver} * V_{liver} \quad \text{[enterohepatic circulation]} \qquad [8]$$

with A_{Lumen} the amount of compound remaining in the gut tract, A_{Fecus} the amount of compound eliminated in the feces, A_{Portal} the amount of compound distributed to the liver, $A_{Biliary}$ the amount of compound circulated back to intestine, and INTAKE the rate of compound intake after oral bolus (mg/hr).

Liver compartment

$$\frac{dA_{liver}}{dt}(mg/hour) = R_{PLASMA} * Q_{Liver} * (C_{Plasma} - CV_{Liver}) + \frac{dA_{portal}}{dt} - \frac{dA_{Metabolism}}{dt} - \frac{dA_{biliary}}{dt}$$

[9]

$$C_{liver}(mg/l) = A_{liver}/V_{liver} \quad \text{[Concentration in liver]} \qquad [10]$$

$$CV_{liver}(mg/l) = C_{liver}/Kp_{liver}$$ [Concentration leaving liver into blood circulation] [11]

$$\frac{dA_{metab}}{dt}(mg/hour) = K_{Metab} * CV_{Liver}$$ [12]

with A_{metab} the amount of compound metabolized in the liver.

Kidney compartment

$$\frac{dA_{kidney}}{dt}(mg/hour) = R_{PLASMA} * Q_{kidney} * (C_{Plasma} - CV_{kidney}) - \frac{dA_{urine}}{dt}$$ [13]

$$C_{kidney}(mg/l) = A_{kidney}/V_{kidney}$$ [Concentration in kidney] [14]

$$CV_{kidney}(mg/l) = C_{kidney}/Kp_{kidney}$$ [Concentration leaving kidney] [15]

$$\frac{dA_{urine}}{dt}(mg/hour) = K_{urine} * CV_{kidney}$$ [16]

with A_{urine} the amount of compound excreted into urine.

Yolk-sac placenta and transfer to fetus

$$\frac{dA_{YPla}}{dt}(mg/hour) = R_{PLASMA} * Q_{YPla} * (C_{Plasma} - CV_{YPla}) - \frac{dA_{Yolk\_to\_fetus}}{dt} + \frac{dA_{Fetus\_to\_Yolk}}{dt}$$ [17]

$$C_{YPla}(mg/l) = A_{YPla}/V_{YPla}$$ [Concentration in yolk-sac placenta] [18]

$$CV_{YPla}(mg/l) = C_{YPla}/Kp_{YPla}$$ [Concentration leaving yolk-sac placenta] [19]

$$\frac{dA_{Yolk\_to\_fetus}}{dt}(mg/hour) = k_{placenta\_to\_fetus} * CV_{YPla}$$ [20]

with $A_{Yolk\_to\_Fetus}$ the amount of compound transferred from placenta into fetus.

Chorioallontoic placenta and transfer to fetus

$$\frac{dA_{CPla}}{dt}(mg/hour) = R_{PLASMA} * Q_{CPla} * (C_{Plasma} - CV_{CPla}) - \frac{dA_{Chorio\_to\_fetus}}{dt} + \frac{dA_{Fetus\_to\_Chorio}}{dt}$$ [21]

$$C_{CPla}(mg/l) = A_{CPla}/V_{CPla}$$ [Concentration in chorioallontoic placenta] [22]

$$CV_{CPla}(mg/l) = C_{CPla}/Kp_{CPla}$$ [Concentration leaving chorioallontoic placenta] [23]

$$\frac{dA_{Chorio\_to\_fetus}}{dt}(mg/hour) = k_{placenta\_to\_fetus} * CV_{CPla} \quad [24]$$

with $A_{Chorio\_to\_Fetus}$ the amount of compound transferred from placenta to fetus.

Fetus and transfer to Yolk-sac placenta

$$\frac{dA_{YFetus}}{dt}(mg/hour) = \frac{dA_{Yolk\_to\_fetus}}{dt} - \frac{dA_{Fetus\_to\_Yolk}}{dt} \quad [25]$$

$$C_{YFetus}(mg/kg) = A_{YFetus}/BW_{Fetus}$$ [Fetal concentration from yolk-sac placenta] [26]

$$CV_{YFetus}(mg/kg) = C_{YFetus}/Kp_{Fetus} \quad [27]$$

$$\frac{dA_{Fetus\_to\_Yolk}}{dt}(mg/hour) = k_{Fetus\_to\_Placenta} * CV_{YFetus} \quad [28]$$

with $A_{Fetus\_to\_Yol}$ the amount of compound transferred from fetus to placenta compartment.

Fetus and transfer to Chorioallontoic placenta

$$\frac{dA_{CFetus}}{dt}(mg/hour) = \frac{dA_{Chorio\_to\_fetus}}{dt} - \frac{dA_{Fetus\_to\_Chorio}}{dt} \quad [29]$$

$$C_{CFetus}(mg/kg) = A_{CFetus}/BW_{Fetus}$$ [Fetal concentration from chorioallontoic placenta] [30]

$$CV_{CFetus}(mg/kg) = C_{CFetus}/Kp_{Fetus} \quad [31]$$

$$\frac{dA_{Fetus\_to\_Chorio}}{dt}(mg/hour) = k_{Fetus\_to\_Placenta} * CV_{CFetus} \quad [32]$$

with $A_{Fetus\_to\_Chorio}$ the amount of compound transferred from fetus to placenta compartment.

$$C_{Fetus}(mg/kg) = C_{YFetus} + C_{CFetus}$$ [total concentration in fetus] [33]

For all simulations, Kp_{Fetus} was set to 1, i.e. $C_{YFetus}=CV_{YFetus}$ and $C_{CFetus}=CV_{CFetus}$. The PBK model implementation and simulations were carried out with Berkeley Madonna Windows (Version 8.3.23) using the Rosenbrock Algorithm for stiff systems.

Appendix 1.3 Physiological parameters for PBK modeling

Anatomical and physiological parameters were retrieved from reference-values for laboratory animals and literature (summarized in Table S1). Growth-related body weight (BW) changes in non-pregnant dams were estimated from our data and expressed as function of the gestational day (GD):

$BW_{non\text{-}pregnant}(GD)\ [kg] = BW_{GD0} + 0.002429 * GD$ [34]

with the body weight at GD0 (BW_{GD0}) set to 0.197 kg. Similarly, we estimated the average body weight of the fetus as

$BW_{fetus}(GD)\ [g] = 5.092*10^{-6} * EXP(0.6413*GD)$ [35]

Temporal changes in the blood volume, tissue volume, and blood flow rates of tissues with ongoing pregnancy were chosen according to O'Flaherty et al. (1992) and You et al. (1999), the

total BW of the pregnant dam was calculated based on the BW of non-pregnant dams (Equation 34) and corrected by the dynamic changes in the maternal organs due to pregnancy and the BW of the concepti (fetus and placenta) (Appendix 1.1). The number of concepti (xN) was set to 10.

Appendix 1.4: Physicochemical and kinetic model parameters

Compound (incl. metabolites M1 & M2)	MW	log K_{ow}[c]	Unbound plasma fraction (%)	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)	Metabolism rate in liver: Total[n] (L/hr)	Metabolism rate in liver: Metabolite[o] (L/hr)	Tissue:plasma partition coefficients[a,m]				
				K_{AS}	$K_{Biliary}$	K_{Feces}	K_{Urine}	K_{Metab}		adipose	kidney	liver	rapidly (heart)	slowly (muscle)
Procymidone		3.08	3.0 [e]	0.3	-	0.04	0.08	0.3		2.7	4.4	4.8	3.7	2.7
Vinclozolin	286.11	3.10	59 [d]	0.5	-	-	0.05	1.4		54.6	6.9	7.5	5.8	4.3
M1[i]	304.13	3.52	22 [f]	-	-	-	0.2	-[b]	2.8 [g]	10	8.3	9.1	7.0	5.1
M2[j]	260.11	2.95	30 [f]	-	-	-	0.2	-[b]	0.9 [g]	3.4	6.2	6.7	5.2	3.8
Linuron	249.09	3.20	10.5 [d]	0.5	-	0.08	0.05	1.0		11.9	5.10	5.50	4.30	3.10
M1[k]	235.1	2.70	28 [f]	-	-	-	0.05	0.02	0.22	3.8	4.90	5.30	4.20	3.10
M2[l]	205.04	2.00	52 [f]	-	-	-	0.05	-[b]	0.25	0.7	2.00	2.10	1.80	1.40
Fludioxonil	248.19	4.12	0.5 [d]	1	-	3	0.01	0.3		1.9	5.9	6.4	4.9	3.6
Cyprodinil	225.29	4.00	0.5 [d]	1	1.6	1	0.2	0.01		1.7	5.8	6.4	4.9	3.5
Dimethomorph	387.87	2.68	9.4 [d]	1	-	2	0.01	0.3		3.4	3.3	3.6	2.8	2.1
Imazalil	297.18	3.82	4.8 [d]	1	-	0.5	0.53	-[b]		13.5	5.9	6.5	5.0	3.6
Quinoxyfen	308.14	4.66	5.0 [d]	1	1.4	2	0.01	0.3		23.0	6.3	6.9	5.3	3.8
Fenhexamide	302.2	3.51	5.7 [d]	1	-	3	0.05	0.15		7.6	5.5	6.1	4.7	3.4
o-Phenylphenol	170.21	3.09	4.1 [d]	1	-	0.1	0.01	0.2		3.7	4.5	4.9	3.8	2.8
λ -Cyhalothrin	449.85	6.80	1.0 [f]	1	-	4	0.05	0.3		24.8	6.4	7.0	5.4	3.9
Pyrimethanil	199.25	2.84	2.0 [d]	1	-	0.1	0.01	0.8		1.1	3.6	3.9	3.1	2.3

[a] estimated according to Poulin and Theil (2002); [b] separation into urinary and metabolic elimination was not possible; [c] Collected from EPI Suite (US EPA 2012), when available, experimental data were preferred over modeled data; [d] Wetmore et al. (2012); [e]World Health Organization WHO and FAO) (2007); [f] predicted by pkCSM (2018, http://biosig.unimelb.edu.au/pkcsm/); [g] Sierra-Santoyo et al. (2008); [i] 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid; [j] 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide; [k] 1-(3,4-Dichlorophenyl)-3-methoxyurea; [l] 1-(3,4-Dichlorophenyl)urea; [m] Tissue:plasma partition coefficient for fetus was set to 1; [n] Metabolic clearance; [o] Conversion of parent compound into active metabolite.

Appendix 1.5: Sensitivity analysis

A local parameter sensitivity analysis was performed to identify kinetic model parameters that primarily influenced the average fetal concentrations of the parental compound. Each model parameter was increased by 5% in turn keeping the other ones constant (Chiu et al. 2007). Normalized Sensitivity Coefficients (NSCs) were calculated as

$$NCS = \frac{R^C - R}{P^C - P} * \frac{\mathrm{P}}{\mathrm{R}}$$

where R is the initial value of the response variable (AUC_{Fetus}) and R^C the output after a 5% change. P is the initial value of the model parameter of interest (e.g., renal elimination rate) and P^C is the parameter value modified by an increase of 5 %. The sensitivity analysis was conducted on model simulations for 50mg/kg/day linuron (incl. two main active metabolites), 40mg/kg/day vinclozolin (incl. two main active metabolites) and 40mg/kg/day procymidone, with bi-directional transfer rates between placenta compartments and fetus set to 2 L/hr.

Linuron incl. M1 and M2 metabolites

				parent compound				M1 metabolite				M2 metabolite			
	model parameter	value P	Value P^C	AUC	AUC^C	NCS	NCS %	AUC	AUC^C	NCS	NCS %	AUC	AUC^C	NCS	NCS %
parent unbound plasma fraction	fub	0.105	0.11025	2.278	2.278	0.0005	0.05%	8.969	8.968	-8E-04	-0.08%	17.992	17.992	-0.0008	-0.08%
Blood/Plasma ratio	R	0.55	0.5775	2.278	2.288	0.0868	8.68%	8.969	8.966	-0.005	-0.52%	17.992	17.987	-0.0053	-0.53%
portal GI adsorption	K_{AS}	0.5	0.525	2.278	2.293	0.1304	13.04%	8.969	9.028	0.132	13.15%	17.992	18.111	0.1319	13.19%

Biliary excretion	$K_{Biliary}$	--													
fecal excretion	K_{Feces}	0.08	0.084	2.278	2.262	-0.137	-13.75%	8.969	8.907	-0.137	-13.69%	17.992	17.869	-0.1371	-13.71%
Urine excretion	K_{Urine}	0.05	0.0525	2.278	2.267	-0.097	-9.66%	8.969	8.960	-0.02	-1.96%	17.992	17.974	-0.0198	-1.98%
Metabolic elimination	K_{Metab}	1	1.05	2.278	2.205	-0.643	-64.26%	8.969	8.681	-0.642	-64.24%	17.992	17.414	-0.6423	-64.23%
placenta to fetus	K_{Trans1}	2	2.1	2.278	2.392	0.9971	99.71%	8.969	9.415	0.996	99.64%	17.992	18.888	0.9955	99.55%
placenta to fetus	K_{Trans2}	2	2.1	2.278	2.170	-0.95	-94.98%	8.969	8.543	-0.949	-94.92%	17.992	17.139	-0.9485	-94.85%
M1 metabolite															
Metabolic conversion from parent	K_{CHANGE_M1}	0.22	0.231	2.278	2.262	-0.145	-14.47%	8.969	9.349	0.848	84.75%	17.992	17.862	-0.1451	-14.51%
Blood/Plasma ratio	R	0.55	0.5775	2.278	2.278	9E-05	0.01%	8.969	8.943	-0.056	-5.63%	17.992	17.992	-0.0004	-0.04%
Urine excretion	K_{Urine}	0.05	0.0525	2.278	2.278	-5E-04	-0.05%	8.969	8.721	-0.553	-55.30%	17.992	17.992	-0.0006	-0.06%
Metabolic elimination	K_{metab_M1}	0.02	0.021	2.278	2.278	-4E-04	-0.04%	8.969	8.821	-0.33	-32.99%	17.992	17.992	-0.0006	-0.06%
M1 metabolite															
Metabolic conversion from parent	K_{CHANGE_M2}	0.25	0.2625	2.278	2.259	-0.165	-16.46%	8.969	8.895	-0.165	-16.48%	17.992	18.736	0.827	82.70%
Blood/Plasma ratio	R	0.55	0.5775	2.278	2.278	0	0.00%	8.969	8.968	-4E-04	-0.04%	17.992	17.872	-0.1337	-13.37%
Urine excretion	K_{Urine}	0.05	0.0525	2.278	2.278	9E-05	0.01%	8.969	8.968	-2E-04	-0.02%	17.992	17.275	-0.7972	-79.72%
Metabolic elimination	K_{metab_M2}	--													

Vinclozolin incl. M1 and M2 metabolites

				parent compound				M1 metabolite				M2 metabolite			
	model parameter	value P	Value P^{c}	AUC	AUC^{c}	NCS	NCS %	AUC	AUC^{c}	NCS	NCS %	AUC	AUC^{c}	NCS	NCS %
parent															
unbound plasma fraction	fub	0.59	0.6195	0.5371	0.5369	-0.011	-1.08%	7.7363	7.736	-1E-04	-0.01%	2.507	2.5065	-0.0003	-0.03%
Blood/Plasma ratio	R	0.55	0.5775	0.5371	0.5397	0.0951	9.51%	7.7363	7.736	-0.001	-0.13%	2.507	2.5064	-0.0013	-0.13%
portal GI adsorption	K_{AS}	0.5	0.525	0.5371	0.5371	-0.001	-0.14%	7.7363	7.736	-3E-04	-0.03%	2.507	2.5065	-0.0003	-0.03%
Biliary excretion	$K_{Biliary}$	--													
fecal excretion	K_{Feces}	--													
Urine excretion	K_{Urine}	0.05	0.0525	0.5371	0.5349	-0.083	-8.28%	7.7363	7.734	-0.006	-0.59%	2.507	2.5058	-0.006	-0.60%
Metabolic elimination	K_{Metab}	1.4	1.47	0.5371	0.5299	-0.269	-26.90%	7.7363	7.632	-0.269	-26.86%	2.507	2.4729	-0.2687	-26.87%
placenta to fetus	K_{Trans1}	2	2.1	0.5371	0.5639	0.9975	99.75%	7.7363	8.122	0.9976	99.76%	2.507	2.6316	0.99738	99.74%
placenta to fetus	K_{Trans2}	2	2.1	0.5371	0.5116	-0.95	-94.98%	7.7363	7.369	-0.949	-94.94%	2.507	2.3876	-0.9495	-94.95%
M1 metabolite Metabolic conversion from parent	K_{CHANGE_M1}	2.8	2.94	0.5371	0.5229	-0.531	-53.05%	7.7363	7.908	0.4432	44.32%	2.507	2.4401	-0.5304	-53.04%
Blood/Plasma ratio	R	0.55	0.5775	0.5371	0.5372	0.0007	0.07%	7.7363	7.745	0.023	2.30%	2.507	2.5067	0.00096	0.10%
Urine excretion	K_{Urine}	--													
Metabolic elimination	K_{metab_M1}	0.2	0.21	0.5371	0.5372	0.0003	0.03%	7.7363	7.371	-0.944	-94.37%	2.507	2.5066	0.00048	0.05%
M1 metabolite Metabolic conversion from parent	K_{CHANGE_M2}	0.9	0.945	0.5371	0.5325	-0.173	-17.32%	7.7363	7.669	-0.173	-17.30%	2.507	2.6091	0.81833	81.83%

Blood/Plasma ratio	R	0.55	0.5775	0.5371	0.5372	0.0004	0.04%	7.7363	7.737	0.0005	0.05%	2.507	2.5089	0.01875	1.88%
Urine excretion	K_{Urine}	--													
Metabolic elimination	K_{metab_M2}	0.2	0.21	0.5371	0.5371	-4E-04	-0.04%	7.7363	7.736	-2E-04	-0.02%	2.507	2.3877	-0.9482	-94.82%

Procymidone

				parent compound			
	model parameter	**value P**	**Value P[C]**	**AUC**	**AUC[C]**	**NCS**	**NCS %**
parent							
unbound plasma fraction	fub	0.03	0.0315	6.17793	6.17803	0.000323733	0.03%
Blood/Plasma ratio	R	0.55	0.5775	6.17793	6.19928	0.069117002	6.91%
portal GI adsorption	K_{AS}	0.3	0.315	6.17793	6.21217	0.11084619	11.08%
Biliary excretion	$K_{Biliary}$	--					
fecal excretion	K_{Feces}	0.04	0.042	6.17793	6.14181	-0.11693237	-11.69%
Urine excretion	K_{Urine}	0.08	0.084	6.17793	6.11884	-0.19129385	-19.13%
Metabolic elimination	K_{Metab}	0.33	0.3465	6.17793	5.92368	-0.82309123	-82.31%
placenta to fetus	K_{Trans1}	2	2.1	6.17793	6.48617	0.997874693	99.79%
placenta to fetus	K_{Trans2}	2	2.1	6.17793	5.88475	-0.9491205	-94.91%

Appendix 2. *In vitro* and background data

Appendix 2.1 Toxicity data for QIVIVE approach on nine pesticides

	***In vitro* activity**		***In vivo* toxicity**		
			One- or Two-generation rat studies on pregnant dams and pups[a]		
	AR antagonism (IC_{20} µM)[c]	Testosterone inhibition[d] (IC_{20} µM)	NOAEL (mg/kg bw)	LOAEL (mg/kg bw)	Critical toxicity
Fludioxonil	0.8	1.3	21	212	Reduced maternal and pup BW
Cyprodinil	15.1(1.91)	0.4	71	292	Reduced maternal and pup BW
Dimethomorph	0.3	2.8	20	67	Reduced maternal and pup body weight, reduced gestation length
Imazalil	3.2	0.5	20	80	Increased duration of gestation , effects on pups/fetuses related to maternal toxicity
Quinoxyfen	4.8	1.0	25	100	Reduced pup BW during lactation, liver hypertrophy
Fenhexamide	2.0	5.2	45	450	Reduced maternal body and organ weight
O-Phenylphenol	3.4	25	40	140	Maternal BW gain, bladder & kidney effect
λ-Cyhalothrin	23.1	NE	0.5[b]	1.5[b]	Reduced maternal and pup BW
Pyrimethanil	27.2 (27.8)	NE	23	294	Reduced maternal and pup BW

[a] data obtained from EFSA's Draft Assessment Reports (DARs), peer review reports available through https://www.efsa.europa.eu/en/publications; [b] values are for cyhalothrin; [c] Orton et al. (2011), in brackets IC_{20} for interfering androgenicity; Testosterone inhibition was measured in NCI-H295R cells. Table taken from Scholze et al. 2020.

Apendix 2.2 Overview of pesticide-induced effects on steroidogenesis in H295R cells *in vitro*

Concentration-response trends observed in NCI-H295R cells are indicated by arrows and the lowest observed effect concentrations (LOECs, µM) are shown in parentheses.

	Testos-terone	Andro-stendi-one	DHEA	OH-Pro-geste-rone	Proge-sterone	Estra-diol	Corti-coste-rone	Corti-sol
Fludioxonil	↓(1.6)	↓(25)[a]	↔	↓(3.1)	↔	↑(3.1)	↑(12.5)[a]	↔
Cyprodinil	↓(6.3)	↓(6.3)	↔	↔	↔	↑(6.3)	↑(25)	↑(25)
Dimethomorph	↔	↔	↓(25)	↓(12.5)	↔	↑(0.8)	↑(0.8)	↔
Imazalil	↓(0.8)	↓(0.8)	↓(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)	↓(25)	↓(12.5)	↑(12.5)	↓(3.1)	↓(3.1)
o-Phe-nylphenol	↓(50)	↔	↔	↔	↔	↑(25)	↔	↔
λ-Cyhalothrin	↔	↔	↔	↔	↔	↔	↑(3.1)[c]	↑(3.1)[c]
Pyrimethanil	↔	↔	↔	↔	↔	↔	↔	↔

↑ : increased synthesis; ↓ : decreased synthesis; ↔ : no effect; DHEA: Dehydroepiandrosterone; [a] cytotoxicity was observed at concentrations >12.5 µM; [b]: cytotoxicity was observed at the same concentrations; [c] : increase starting at 3.1µM, decrease from 25 µM. Table taken from Scholze et al. 2020.

Appendix 2.3 Comparison of potencies of pesticides on prostaglandin synthesis D2 inhibition in vitro and effects on anogenital distance in vivo

Pesticides	PGD2 inhibition (IC50, nM)[a]	In vivo AGD effect
O-Phenylphenol	175	?
Cyprodinil	803	Yes
Linuron	1490	Yes
Imazalil	1510	No
Fenhexamid	7370	?
Methiocarb	7850	?
Pyrimethanil	8270	?
Fludioxonil	30200	Yes
Dimethomorph	inactive	Yes

Appendix 3. *In vivo* study reports

Appendix 3.1 Effects on dams and offspring after gavage of three doses of Fludioxonil, Cyprodinil and Dimethomorph in Wistar rats on gestation day (GD) 7–21 and postnatal days (PD) 1–16

	Control	**Fludioxonil** (mg/kg bw/day)			**Cyprodinil** (mg/kg bw/day)			**Dimethomorph** (mg/kg bw/day)		
		20	**60**	**180**	**20**	**60**	**180**	**6.7**	**20**	**60**
No. of dams (litters born)	12(8)	10(10)	10(8)	10(6)	10(9)	10(9)	10(7)	10(8)	10(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 length (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
AGD index males	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
AGD index females	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
Offspring 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
Offspring 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). Table taken from Scholze et al. 2020.

Appendix 3.2 Report on *in vivo* dimethomorph studies

Overview of the design of *in vivo* developmental studies with dimethomorph

Study design	Dose levels (no. of dams per dose)	Endpoints in adults/dams	Endpoints studied in offspring PD1-14
Range-finding study in pregnant animals,	Control and 2 doses Doses: 120;180 mg/kg/day (N = 4 dams)	Body weight gain during pregnancy and lactation Gestation length Litter size Dystocia	Birth weight, growth and survival Anogenital distance and nipple retention
Dose response study in pregnant animals (Study 4 in Scholze et al., 128 (11): 117005, 2020)	Control and 4 doses: 6.7, 20, 60, 180 mg/kg/day (N= 10-11 dams; N=5 for GD 21 section)	Body weight gain during pregnancy and lactation Gestation length Litter size Dystocia	Birth weight, growth and survival Anogenital distance and nipple retention, Weight and histopathology of reproductive organs Chemicals and hormones in blood Chemicals in milk

Effects on dams and offspring after gavage of four doses of dimethomorph in Sprague Dawley rats on GD7–21 and PD1–16

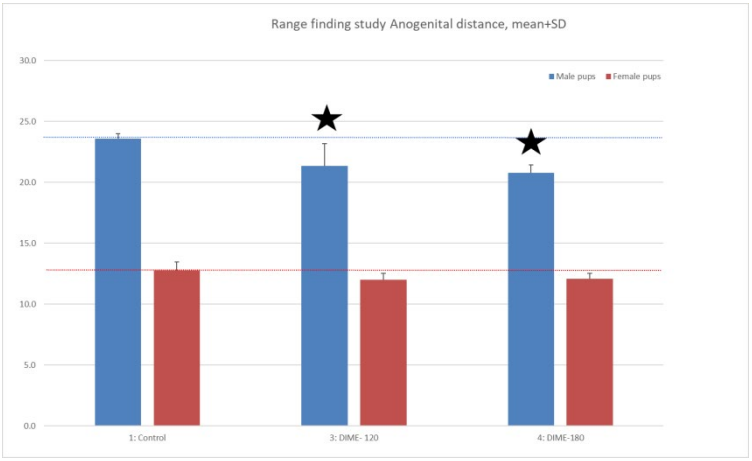
	Control	**Dimethomorph** (mg/kg/bw)			
		6.7	**20**	**60**	**180**
No. of dams (litters)	12(11)	10(10)	11(11)	11(11)	11(10)
Dam bw gain,GD7-21 #	115.6± 12.8	116.9 ± 19.2	119.5 ± 11.7	109.9 ± 19.5	104.8 ± 20.1
Dam bw gain, GD7- PD 1	33.4 ± 12.7	31.1 ± 16.0	34.6 ± 11.0	34.2 ± 15.5	27.5 ± 20.2
Dam bw gain PD 1-14	33.6 ± 15.7	27.5 ± 10.3	39.4 ± 5.5	39.7 ± 11.0	44.9 ± 16.8
Gestation length (days)	23.0 ± 0.0	23.0 ± 0.5	23.0 ± 0.0	22.8 ± 0.4	22.9 ± 0.6
% postimplantation loss	5.0 ± 6.1	5.6 ± 6.3	2.6 ± 4.9	4.3 ± 5.2	10.1 ± 12.6
% perinatal loss	5.6 ± 5.9	7.5 ± 7.2	3.5 ± 6.3	5.7 ± 6.0	13.3 ± 11.8
Litter size	14.2 ± 1.9	14.5 ± 3.1	13.8 ± 2.0	12.9 ± 2.1	12.7 ± 2.9
% postnatal deaths	0.6 ± 1.9	1.9 ± 5.9	1.6 ± 3.7	1.4 ± 3.1	3.2 ± 7.2
Offspring					
Male birth weight	7.06 ± 0.5	6.83 ± 0.7	7.24 ± 0.5	6.68 ± 0.6	6.49 ± 0.9
AGD males (units)	23.27 ± 0.6	23.56 ± 0.7	22.83 ± 1.0	**21.39 ± 1.0****	**21.31 ± 1.7****
AGD males (mm)	3.82 ± 0.1	3.86 ± 0.1	3.74 ± 0.2	**3.51 ± 0.2****	**3.57 ± 0.2****
AGD index males	1.99 ± 0.1	2.04 ± 0.1	1.94 ± 0.1	**1.87 ± 0.1****	**1.90 ± 0.11****
Nipple retention males	0.2 ± 0.2	**0.8 ± 0.7****	0.4 ± 0.5	0.6 ± 0.8	**2.2 ± 2.0****
Female birth weight	6.7 ± 0.5	6.4 ± 0.7	6.8 ± 0.4	6.3 ± 0.6	6.2 ± 0.9
AGD females (units)	12.6 ± 0.6	13.1 ± 0.3	12.4 ± 0.8	11.8 ± 0.6	12.1 ± 0.9
AGD females (mm)	2.1 ± 0.1	2.2 ± 0.0	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.2
AGD index females	1.1 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	1.1 ± 0.0
Nipple retention females	12.1 ± 0.2	12.0 ± 0.1	12.0 ± 0.1	12.1 ± 0.1	12.0 ± 0.1
Offspring weight PD 6 males	12.7 ± 2.0	11.8 ± 1.2	13.0 ± 0.9	13.0 ± 1.2	12.6 ± 1.7
Offspring weight PD 14 males	26.5 ± 4.8	24.0 ± 2.5	27.6 ± 3.6	26.8 ± 2.5	27.6 ± 3.3

Two pregnant rats were killed during gestation due to maternal toxicity from control and 180 group respectively. # Dam BW gain was calculated for dams giving birth to viable litters. Data represents mean ± SD. Bold values are statistically significant compared to controls (* p<0.05; ** p<0.01). Table taken from Scholze et al. 2020.

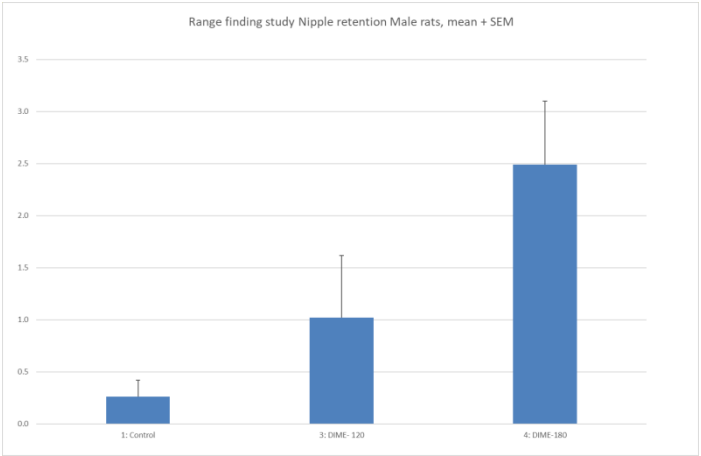
In the range-finding study on dimethomorph the 180 mg/kg bw/day dose indicated mild maternal toxicity (increased post-implantation and perinatal loss, higher postnatal death rate), but not any dystocia (disrupted parturition) or severe toxicity. Therefore, the high dose of 180 was also included in the final dose response study.

In the range finding study a reduction of male AGD was seen at both doses of 60 and 180 mg/kg/day. Likewise, the final study (study 4) showed statistically significantly reduced male AGDs at doses of 60 and 180 mg/kg/day (Figure 7C, black symbols,). The effect at 60 mg/kg bw/d contradicted the first (lower powered) dimethomorph study (study 3), in which the two lowest doses (6.7 and 20 mg/kg bw/d) led to statistically significantly reduction in male AGD, but no effect was seen at the higher dose of 60 mg/kg/day (Figure 7C, red symbols).

In the range finding study and the final dimethomorph study (study 4) retained nipples were seen at doses of 120 and 180 mg/kg/day and doses of 7 and 180 mg/kg/day, respectively.



Range finding study with dimethomorph (N=4). Significant decrease in AGD was seen in male offspring at both 120 and 180 mg/kg/day.

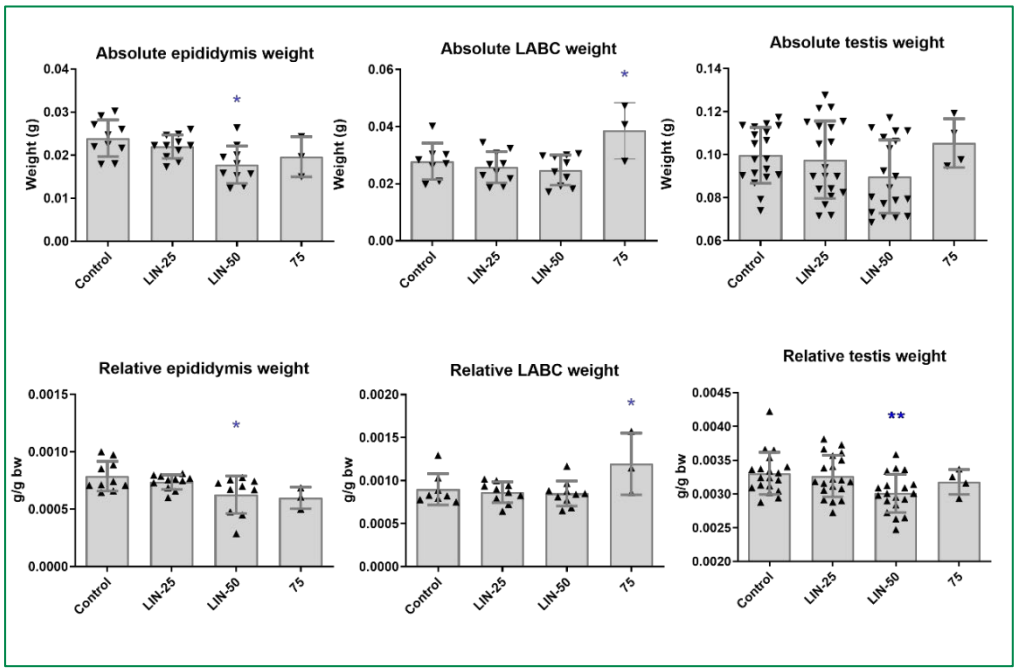


Range finding study with dimethomorph (N=4). Increased number of nipples was seen in male offspring PD14 at both 120 and 180 mg/kg/day.

Appendix 3.3 Report on linuron *in vivo* studies

Overview of the *in vivo* study on Linuron

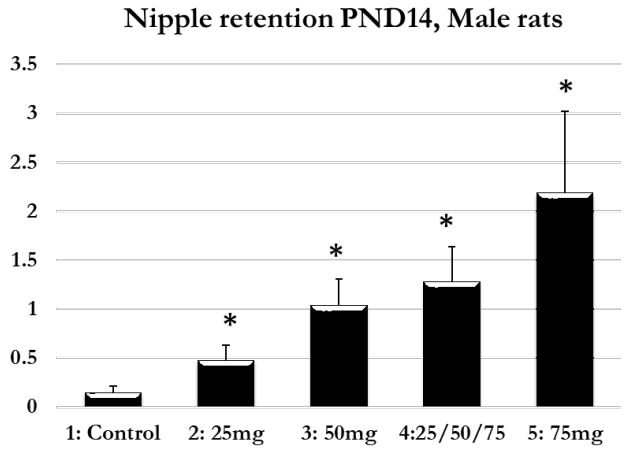
Dose levels in each cohort (no. of animals per dose)	Endpoints in adults/dams	Endpoints studied in offspring, PD1-14
GD17 section: 0, 25, 50, 100 mg/kg bw/d (n=2) GD21 section: 0; 25; 50; 75 mg/kg bw/d (n=3) PD16 section: 0, 25, 50 and 75 mg/kg bw/d (n=10-11, but in high dose group n=3).	Body weight gain during pregnancy and lactation Gestation length Litter size Maternal toxicity and dystocia	Birth weight, growth and survival Weight of reproductive organs and thyroid AGD and nipple retention Chemical levels in blood and milk (PD6) Hormone levels in blood



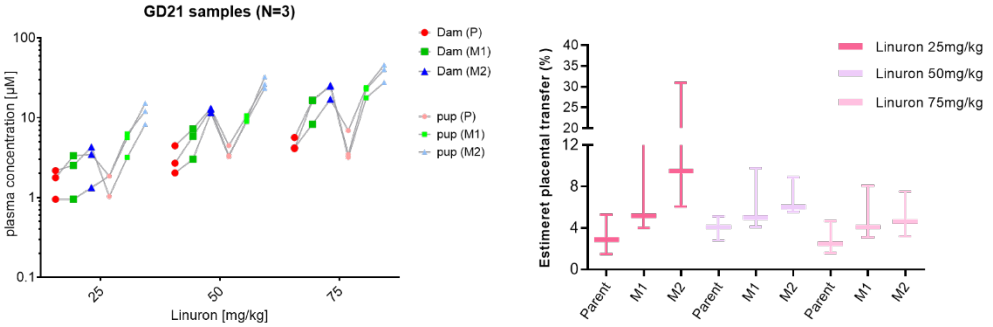
As listed in the methods section, the high doses were reduced due to maternal toxicity. Specifically, we observed weight loss, lethargy and inactivity shortly after dosing.
Weights of male reproductive organs at PD16 were reduced by linuron, whereas body weights were unchanged. Specifically, absolute and relative weight of epididymis was reduced at 50 mg/kg bw/d, whereas absolute and relative weight of LABC muscle was increased at 75 mg/kg bw/d. Relative, but not absolute testis weight was reduced at 50 mg/kg bw/d. No changes in weights of prostate, seminal vesicle or bulbourethral glands were seen, possibly due to large variations in the control group. The weights of male reproductive organs in pre-puberty were expected to be reduced by the perinatal linuron exposure as seen with other potent anti-androgens ((Christiansen et al. 2008; Hass et al. 2007; Ostby et al. 1999; Parks et al. 2000), but only epididymis and testis weight was reduced at the middle dose group. The lack of effect on other reproductive organs may be due to large variations among controls and indicates lower sensitivity of these endpoints compared to AGD and nipple retention.

Effects on linuron on male AGD were measured before and after birth and additional endpoints have been included below. Linuron exposure caused a decrease in AGD in male fetuses (GD21) in all doses and a decrease in AGD in both male and female offspring (PD1) at 25 or

50 mg/kg/day as presented in Figure 3. Moreover, linuron caused a significant increase in nipple retention in the males at PD14 as shown below.

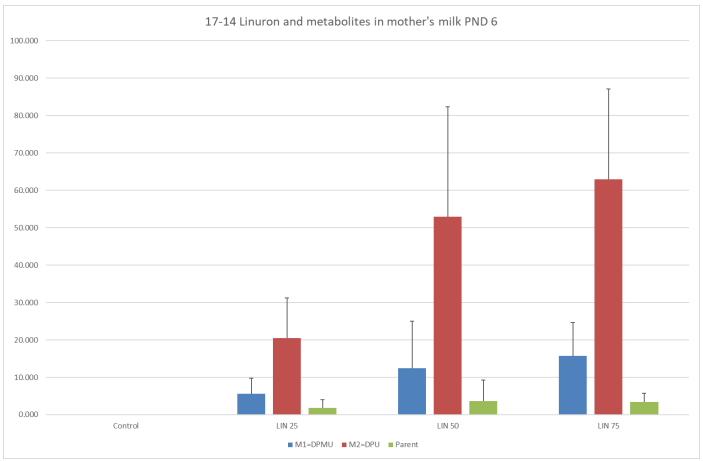


Chemical analysis showed increasing plasma concentrations of the parent compound linuron as well as metabolites (labeled M1 and M2) with increasing doses. Concentrations in pup plasma were comparable or slightly higher than dam plasma concentrations. In particular, the level of the M2 metabolite (DCU – 1-(3,4-dichlorophenyl) urea) is generally higher in the pups than in the dams.



M1= DCXU **M2= DCU**

Linuron and metabolites was measured in mother's milk at PD6. The figure below shows a dose-dependent increase in the amount of both metabolites and parent compound in mother's milk at PD6. M2 DPU (1-(3,4-dichlorophenyl) urea) is present at higher amounts than M1-DPMU and the parent compound.



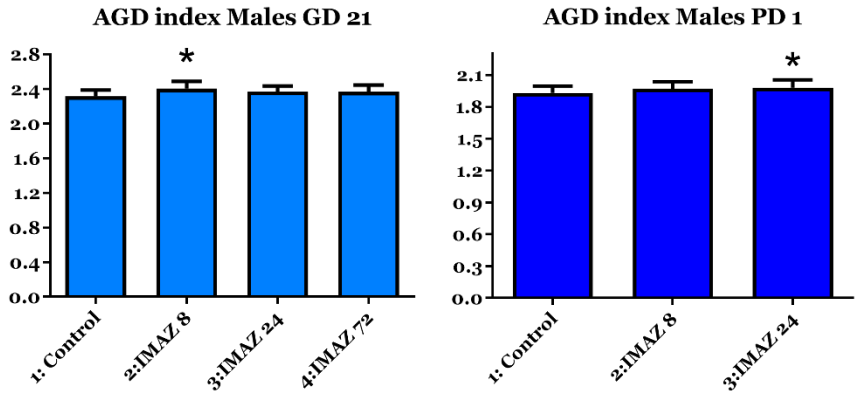
Appendix 3.4 Report on imazalil *in vivo* studies

Overview of the design of *in vivo* developmental studies with imazalil

Study design	Dose levels (number of animals per dose)	Endpoints in adults/dams	Endpoints in offspring PD1-14
Range-finding study in pregnant animals	Control and 3 doses: 40; 55; 70 mg/kg/day (N = 4 dams)	Body weight gain during pregnancy and lactation Gestation length Litter size Dystocia	Birth weight Anogenital distance and nipple retention Growth and survival
Dose response study in pregnant animals	Control and 3 doses: 8, 24, 72 mg/kg/day (N= 13-14 dams; N=3 for GD17 and N=5 for GD21 section)	Body weight gain during pregnancy and lactation Gestation length Litter size Dystocia	Birth weight Anogenital distance and nipple retention Growth and survivalWeight and histopathology of reproductive organs Chemicals and hormones in blood Chemicals in milk

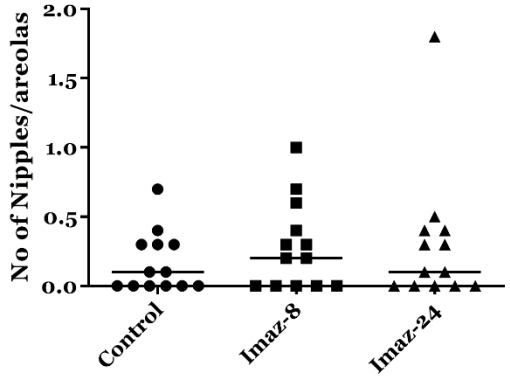
Imazalil has not previously been properly investigated for its endocrine disrupting potential *in vivo*. Based on the compound's anti-androgenic properties *in vitro*, imazalil was initially predicted to reduce male AGD. In summary the range finding study showed that all three tested doses (40, 55 and 70 mg/kg bw/day), caused maternal toxicity and dystocia (disrupted parturition). Therefore, a dose of 24 mg/kg bw/day was chosen as the highest dose to be investigated in the final developmental study for dams that were planned to deliver their offspring. Additionally, a group receiving 72 mg/kg bw /day was included as part of the GD 21 cohort.

In the final study, AGD was measured on GD21 (n=5) and on PD1 (n=13-14) but no indications of decreased male AGD were seen. On GD21, a small but statistically significant increase in male AGD index was seen at 8 mg/kg, but not at the two higher doses. This could be considered a chance finding due to the lack of dose-response and the relatively low number of litters investigated. At PD1, male AGD was not significantly affected at the dose of 8 mg/kg but was significantly increased at 24 mg/kg. The increase was however only 2.5% compared to the controls, and could be related to the fact that male offspring in this dose group had 4% larger body weights compared to controls. This difference in body weight was not statistically significant, but could have affected the AGD index. When analysing the AGD with BW as covariate the finding was not significant. This could indicate insufficient correction (or overcorrection) for the influence of body weight on AGD index.
The incidence of nipple retention (measured on PD13) was similar between all dose groups.



Male AGD index was significantly increased at 8 mg/kg on GD21 (n=5 litters/group) and at 24 mg/kg on PD1 (n= 13-14 litters per group) following in utero exposure to Imazalil.

At PD17 body weights and reproductive organ weights in the perinatally exposed offspring were not affected at the high dose of 24 mg/kg bw/d. At the dose of 8 mg/kg a statistically significant reduction in relative testis weights and an increase on the weight of the LABC muscle were observed. No changes in body weights or weights of prostate, epididymis, seminal vesicle or bulbourethral gland were seen at either dose.



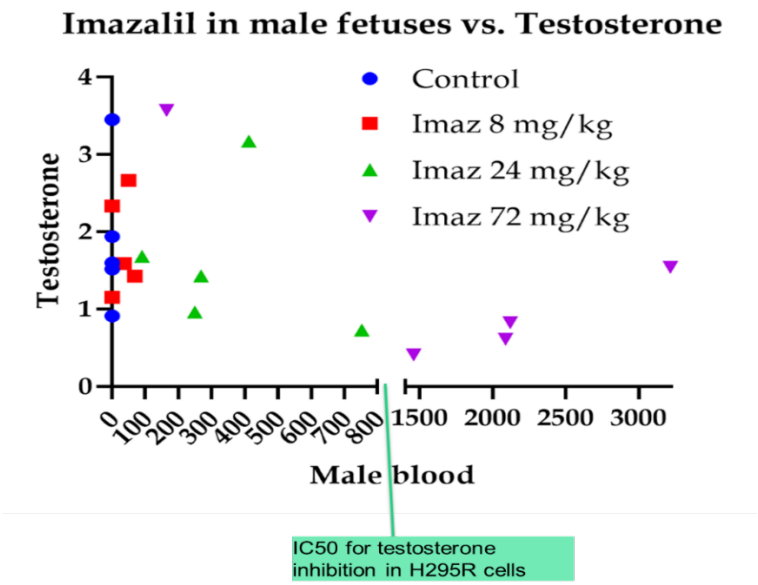
Nipple retention measured at PND 14 (n=14-16 litters/group) was not affected by perinatal exposure to imazalil.

On GD21 internal concentrations of imazalil were measured in plasma from dams and offspring as well as in the amniotic fluid (N=5). The chemical analysis showed dose related increases in imazalil concentration, but with a very high degree of variation at the high dose of 72 mg/kg/day, especially in the male and female fetal plasma samples. The amniotic fluid samples, on the other hand, showed less variation and generally lower imazalil concentration that those present in the fetal plasma.

As imazalil did not reduce male AGD, it was considered relevant to evaluate whether imazalil was able to reduce fetal testosterone levels. Measurement of testicular testosterone content at GD21 showed large variations between dose groups. In the high dose imazalil group (Imaz-72), one sample showed very low imazalil concentrations, whereas four other samples had high imazalil concentrations. The males with very high imazalil exposure levels also had relatively low testosterone concentrations, but these concentrations were comparable to the lower range of testosterone levels in the control group. The few highly exposed animals showed internal exposures above the IC_{50} for testosterone inhibition in H295R cells. Overall, we found no differences between dose groups, even though the fetuses with the highest imazalil concentrations in blood had testosterone concentrations in the lower end of the control range.

We found large variations between individual animals in the high dose group with regard to imazalil concentrations in dam plasma, fetal plasma and amniotic fluid, as well as large variations in intra-testicular testosterone levels. The intra-testicular testosterone levels was not lowered to an extent that resulted in any changes in AGD.

Chemical analysis of pup stomach content showed rather low levels of imazalil indicating low transferred via maternal milk (data not shown).



Intra-testicular testosterone levels and the link to male fetal blood measurements at GD21.

In summary, an anti-androgenic pattern of effects was not observed after developmental exposure to imazalil. Some indications of endocrine disruption were however observed, though subtle, not necessarily dose-dependent and not always in the expected direction.
The difficulties with parturition that the dams experienced at doses above 30 mg/kg bw/day were very likely related to the endocrine disrupting properties of imazalil. Similarly, the small increases in male AGD, and changes in weight of testes and LABC could be signs of a weak androgenic response, at the lower doses of imazalil. The increased gestation length and dystocia have been seen with several other conazole fungicides due to disrupted steroidogenesis including inhibition of aromatase.

Appendix 4. Chemical analyses of pesticides

Reagents and Chemicals

Acetonitrile, methanol, formic acid and 25% ammonium hydroxide (all of LC-MS grade) and ethyl acetate were obtained from Sigma Aldrich, Schneldorf, Germany. Isolute bulk C-18 sorbent was obtained from Biotage, Sweden and was washed with acetonitrile and ethyl acetate and dried prior to use. Water was purified on a Milli Q system, Millipore Corporation, US. Pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), LGC-standards (Teddington, Middlesex, UK) and Sigma Aldrich (St. Louis, MO, USA). Acetonitrile used as solvents for the LC-QTOF was LiChrosolv Hypergrade. An ESI-L low concentration tuning mix from Agilent (Santa Clara, CA, USA) was used for tuning of the TOF. A reference solution was prepared containing Purine and HP0921 from Agilent (Santa Clara, CA, USA).
Stock solutions of the pesticides were prepared at 1 mg/ml in acetonitrile, methanol or toluene. Standard mixtures containing the test compounds were prepared at 10 µg/ml acetonitrile and used for preparation of calibration solutions. Isomers eluting very close to each other were avoided in these mixtures.

Sample preparation

In the fludioxonil, cyprodinil, and dimethomorph studies, 100 µl of amniotic fluid or plasma was added 200 µl of ice-cold acetonitrile in Eppendorf tubes. Internal standard was added in the form of 10 µl TPP (5 µg/ml) and the extraction was performed by whirl mixing. The tube was then stored on ice for 5 min and then centrifuged at 10,000 x g for 10 min at 4 ˚C (Ole Dich Instrument, Denmark). An aliquot of the supernatant was transferred to filter vials (0.45 µm) and analysed by LC-QTOF.
Quality control samples were included in each analytical run. These were prepared using the same procedure as for the samples, though using amniotic fluid from untreated control Wistar rats extracted with acetonitrile containing three different levels of the relevant pesticide standards. The resulting spike levels were 0.04, 0.013 and 0.13 µg/ml.
For the analysis of procymidon, vinclozolin, linuron and selected metabolites, 50 µl of amniotic fluid or plasma from Sprague Dawley rats was transferred to an Eppendorf vial and added 150 µl of acetonitrile. 50 mg of isolute C-18 sorbent was added to the tube, which was shaken for 5 min, cooled to 4 ˚C and centrifuged at 10,000 x g for 10 min at 4 ˚C (Ole Dich Instrument makers, Denmark). 20 µl of the supernatant was transferred to an HPLC vial and mixed with 80 µl of 5%-75% acetonitrile (this percentage was analyte dependent) prior to analysis by LC--QTOF analysis. Pesticide screening was carried out using a liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometer.
The LC system was connected to a Bruker Daltonics, maXis qTOF mass spectrometer equipped with an electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). The ion source settings were: nebulizer pressure 2 bars, drying gas flow 8 l/min, dry gas temperature 200 °C, capillary voltage 2500 V. The scan range was from 80 to 700 m/z with an acquisition rate of 2 Hz. Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2-0.4 min time segment and used for internal calibration of the data files. Hexakisperflouroetoxyphophazene was used as lock mass calibrant.

Matrix matched standard samples were prepared in blank plasma at 4 - 5 different levels. Standards and blanks were analyzed in the beginning of a sequence and after each set of 20 samples.
Data files were processed using QuantAnalysis (Bruker Daltonics, Bremen, Germany). Extracted ion chromatograms of pseudomolecular ions $[M+H]^+$ +/- 0.005 Da were constructed and integrated. If sodium and/or ammonium adducts were observed in the mass spectra, extracted ion chromatograms of these adducts were added to the chromatograms of the pseudomolecular ions. Plasma concentrations were calculated based on linear calibrations curves constructed using 1/x weighing.
The limits of quantification (LOQ) for vinclozolin metabolites, M1 and M2 were 1.5 and 0.2 µM, respectively, for linuron and the metabolites, DCXU and DCU, they were approximately 0.1 µM, for procymidon the LOQ was 0.2 µM. For fludioxonil, cyprodinil and dimethomorph the LOQs were approximately 0.06, 0.06 and 0.04 µM, respectively.

Appendix 5. Overview of JANUS and PANDA project

Table illustrating the project in which data was generated for each pesticide

		PANDA project	JANUS project	Data from the past
In silico	PBK modeling	Fludioxonil Cyprodinil Dimethomorph Imazalil Quinoxyfen Fenhexamid o-Phenylphenol λ-Cyhalothrin Pyrimethanil	Imazalil Procymidone Vinclozolin Linuron	
In vitro	AR antagonism		Procymidone Vinclozolin & metabolites M1, M2 Linuron & three metabolites	Fludioxonil Cyprodinil Dimethomorph Imazalil Quinoxyfen Fenhexamid o-Phenylphenol λ-Cyhalothrin Pyrimethanil
	Androgen synthesis	Fludioxonil Cyprodinil Dimethomorph Imazalil Quinoxyfen Fenhexamid o-Phenylphenol λ-Cyhalothrin Pyrimethanil	Procymidone Vinclozolin Linuron & three metabolites	
	In vitro metabolism			Prochloraz Fludioxonil Imazalil Vinclozolin Procymidone
In vivo	Study on pregnant rats	Fludioxonil incl. long term study on epigenetics & prostate effects Cyprodinil Dimethomorph	Dimethomorph Procymidone Vinclozolin Linuron Imazalil Finasteride	Procymidone Vinclozolin

	Placental transfer		Fludioxonil Cyprodinil Dimethomorph Procymidone Vinclozolin Linuron Imazalil Finasteride	

Towards an improved human risk assessment of pesticides for adverse effects on male reproductive health

Many pesticides can antagonize the androgen receptor (AR) or inhibit androgen synthesis in vitro but their potential to cause reproductive toxicity related to disruption of androgen action during fetal life is difficult to predict. Currently no approaches for utilizing in vitro data to anticipate such in vivo effects exist and limitation of unnecessary in vivo testing is urgently needed.

The aim was to develop a quantitative in vitro to in vivo extrapolation (QIVIVE) approach for predicting in vivo anti-androgenicity arising from gestational exposures and manifesting as a shortened anogenital distance (AGD) in male rats.

We built physiologically-based pharmacokinetic (PBK) models to simulate fetal levels of chemicals resulting from maternal dosing. The predicted fetal levels were compared to measured concentrations, and these were judged against in vitro active concentrations for AR antagonism and androgen synthesis suppression.

We applied the approach to three model pesticides (procymidone, vinclozolin, linuron) and nine current-use pesticides. Our PBK model predicted accurately the measured fetal concentrations of compounds. Seven pesticides (fludioxonil, cyprodinil, dimethomorph, imazalil, quinoxyfen, fenhexamid, o-phenylphenol) and the model pesticides were predicted to produce a shortened AGD in male pups, while two (λ-cyhalothrin, pyrimethanil) were anticipated inactive. We tested these expectations for the model pesticides, fludioxonil, cyprodinil and dimethomorph and observed shortened AGD in male pups after gestational exposure.

Our QIVIVE model newly identified fludioxonil, cyprodinil and dimethomorph as new in vivo anti-androgens. With the examples investigated, our approach shows great promise for predicting in vivo anti-androgenicity for chemicals and for minimizing unnecessary in vivo testing.

The Danish Environmental
Protection Agency
Tolderlundsvej 5
DK - 5000 Odense C

www.mst.dk