

**EFFECTS OF MIXTURES OF PHTHALATES,
PESTICIDES AND TCDD ON SEXUAL
DIFFERENTIATION IN RATS: A RISK FRAMEWORK
BASED UPON DISRUPTION OF COMMON
DEVELOPING SYSTEMS.**



*USEPA scientist
grapples with difficult
environmental issues*

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*This presentation does not necessarily reflect USEPA policy, but rather
represents the author's current view on the state of the science*

Reproductive Toxicology Branch, NHEERL, ORD, USEPA

Potential Developmental Reproductive Toxicants Studied

AR Antagonists

Compete with natural hormones T and DHT for AR, prevent AR-DNA binding in vitro, inhibit AR-dependent gene expression in vivo, and may induce malformations in male reproductive tract and delay puberty in male rat

- Vinclozolin
- Procymidone
- Linuron
- p,p' DDE and other o,p'- and p,p' DDT metabolites
- Prochloraz

Inhibitors of fetal androgen synthesis

Prevent the synthesis of natural hormones T and DHT and induce malformations in male reproductive tract and delay puberty in male rat

- DEHP, DoTP, BrDEHP
- BBP, DBP, DiBP
- DINP
- DiHexyl P, DiCycloHP
- DiHeptylP, DiheptylP
- DP(enty)P
- DEP, DMP
- Linuron
- Prochloraz

Estrogens

Methoxychlor
Ethinyl Estradiol
Bisphenol A

Fetal Germ Cell Toxicants

Busulfan
Diazo dyes

Steroidogenesis inhibitors

Ketoconazole
Fenarimol

Androgens

Testosterone
Trenbolone

Dioxins and PCBs

Dioxin
PCB 169 congener

Objectives of our research

- **Determine how chemicals with similar and dissimilar mechanisms of toxicity interact during sexual differentiation**
- **To provide a framework for deciding what chemicals to include in a cumulative risk assessment**

- *Working Hypothesis:*

Chemicals that disrupt the development of a common reproductive tissue/system during sexual differentiation will produce dose additive responses, regardless of the molecular mechanism or the signaling pathway that is disrupted

Cumulative Risk: Key Question

- Should chemicals that disrupt differentiation of the same reproductive tissue but by different molecular mechanisms of action in different tissues be included in a Common Mechanism Group?

- The default answer is NO

because such a mixture would not be expected to produce adverse effects if each chemical is administered below the NOAEL

Response addition (RA)

- ***$R1+R2+\dots+Ri = \text{No Adverse Effects}$***

- ***However if this assumption is incorrect
Dose addition (DA)***

- ***$R1+R2+\dots+Ri = \text{Malformations}$***

Main points from National Academy of Science report:
*'Phthalates and Cumulative Risk Assessment:
The Task Ahead'*

- **Mixtures of phthalates produce effects that are well-predicted using the dose-addition model.**
- Other antiandrogen mixtures (including mixtures of phthalates with other antiandrogens) also conform to the dose-addition model.
- **The response-addition model is not an appropriate model for antiandrogenic chemicals.**
- The criteria for including chemicals in cumulative risk assessments are too narrow and should be expanded to include chemicals with similar effects.
- **The current cumulative risk assessment process is not protective because it does not include all chemicals, which elicit dose additive toxicity.**

- Hormone dependent endpoints

- Anogenital distance at birth

- Nipple/ areolar numbers in infants

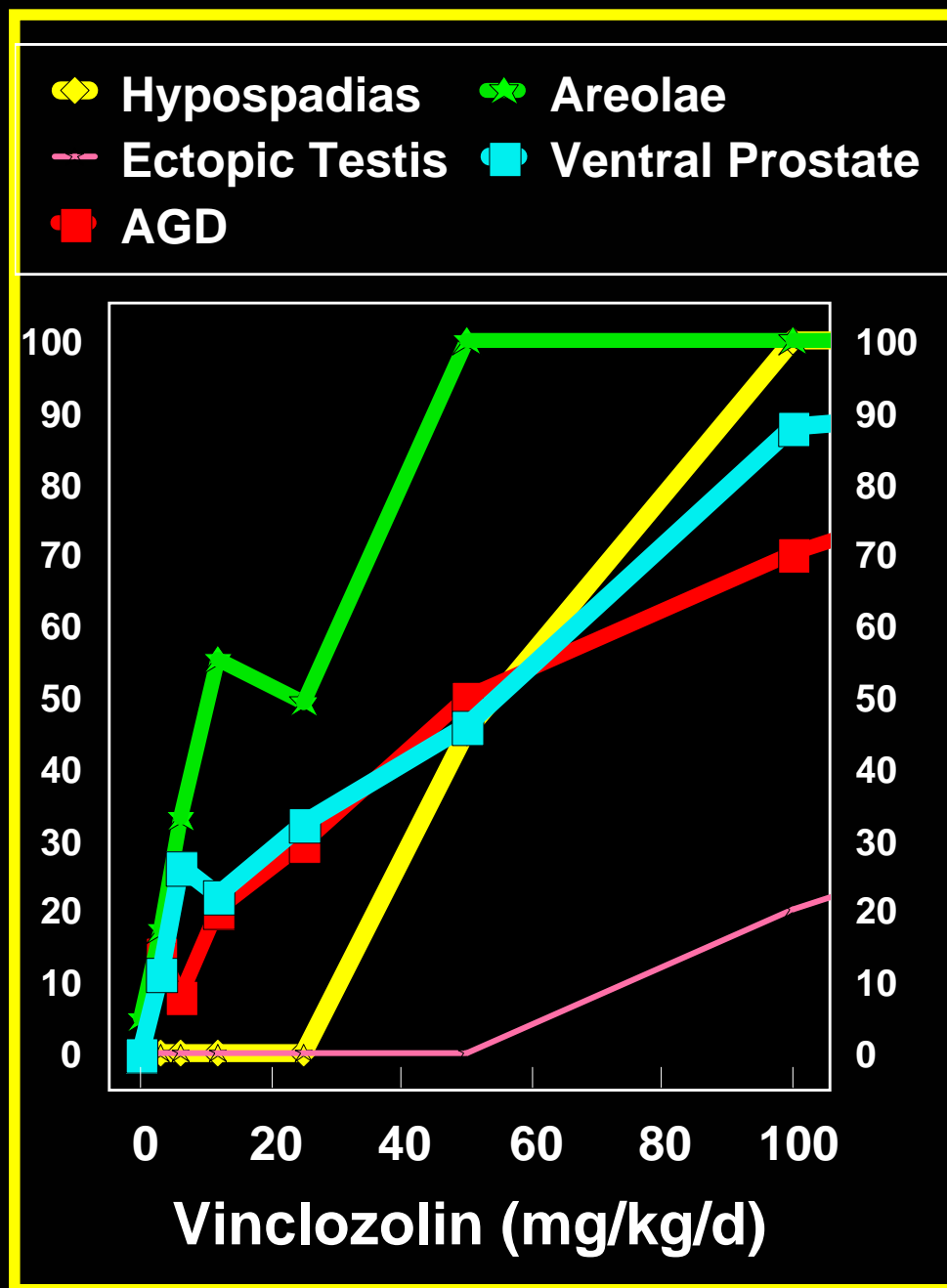
- Reproductive Malformations

- Undescended testes
- Gubernacular abnormalities
- Epididymal agenesis
- Ventral prostate agenesis
- Seminal vesicle agenesis
- Vas deferens agenesis
- Nipples
- Hypospadias
- Vaginal pouch


- Reproductive Organ Weights

- Glans penis
- Ventral prostate
- Seminal vesicle
- Testes
- Epididymides
- Levator ani bulbocavernosus
- Cowper's glands

- Testis and epididymal histopathology



Predictions of mixture effects

- Several methods are being used to predict the “expected” outcome of the mixture on reproductive endpoints.
 - Mixture effects are being predicted from individual chemical dose response data by
 - **Dose Addition**
 - **Response Addition**
 - **Integrated Addition**, a “mixed” model that includes both dose and response addition.
- 

In utero mixture studies

- *Similar cellular and molecular mechanisms of action*
 - Binary mixtures – pairs of pesticides and pairs of phthalates
 - Mixture of 5 phthalates.
 - Mixture of 9 phthalates
 - An AR agonist combined with an AR antagonist
- *Diverse cellular and molecular mechanisms of action*
 - Binary mixtures – pairs of chemicals
 - Mixture of 7 chemicals including pesticides and phthalates
 - Mixture of 10 chemicals
 - Mixture of a phthalate with 2,3,7,8 TCDD (ongoing)
- To date, dose addition modeling best predicts the effects
- **Supported by: NTP, NIEHS/EPA Interagency Cooperative Research Agreement HHS Y1-ES-8014-01; EPA RW75922855-01-0**

Cumulative effects:

Common mechanisms of toxicity



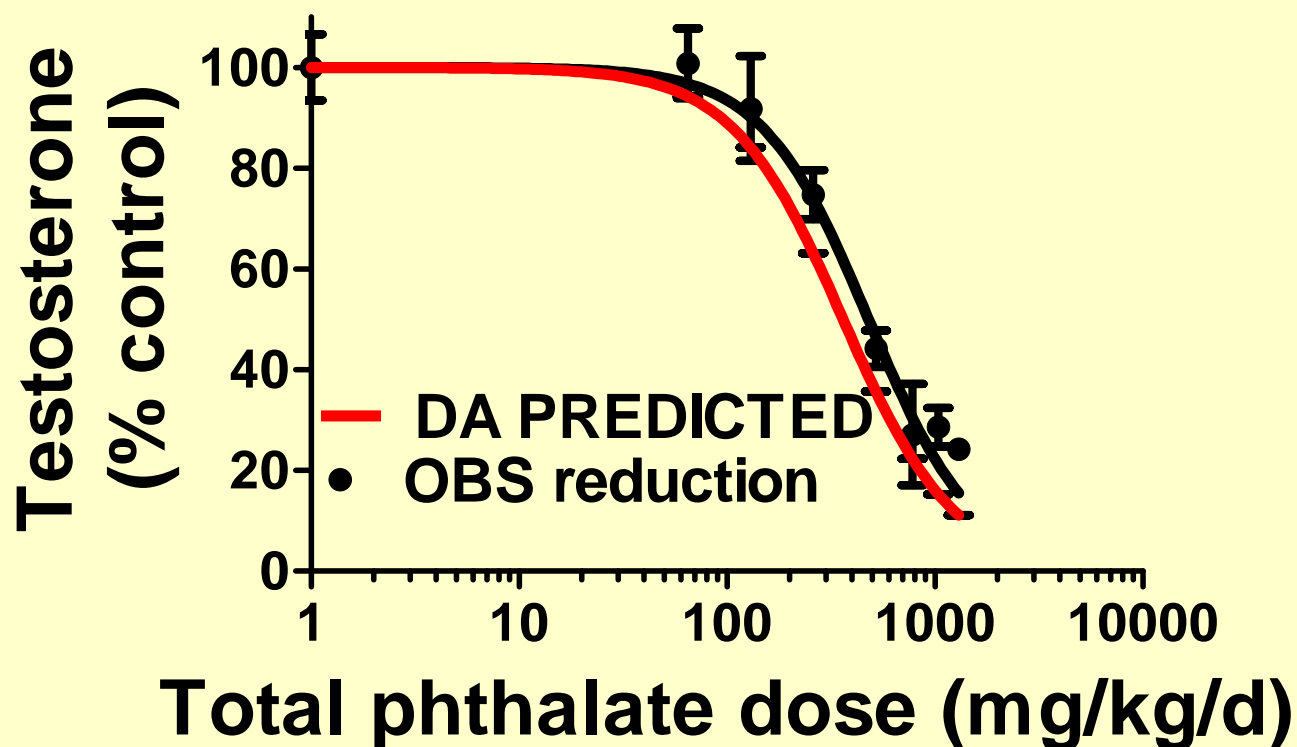
Common mechanism of Toxicity

Effects of a mixture of five phthalates on fetal testosterone production in the rat
(Howdeshell et al, 2008)

Mixture study. Fixed ratio-dilution study

- **DBP, DiBP, BBP, DEHP, DPP** were administered as a mixture GD 8-18 and fetal T production measured on GD 18.
- **Mixture ratio designed so that each phthalate would contribute equally to reduction in fetal T production if the mixture behaved in a dose-additive manner.**

Comparison of the contribution of each of the five individual phthalates with the observed effect of the mixture and the effect predicted (red line) from dose addition modeling



Top dose=300 mg/kg/d four PEs except DPP at 100 mg/kg/d

Phthalates: Independent or Joint Acting Toxicants?

“Sensational Mythology” Or Common Sense?



Cumulative effects:

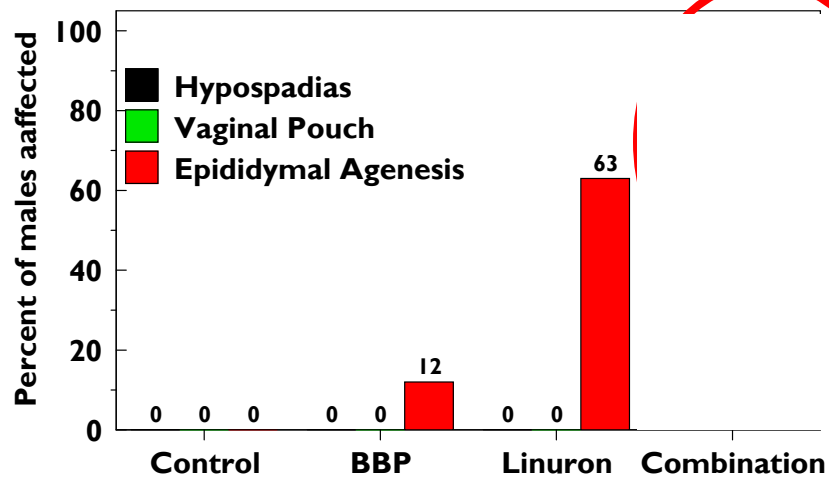
***Diverse mechanisms of toxicity that
disrupt the same signaling pathway***

***Disruption of the Androgen signaling
pathway in fetal tissues***



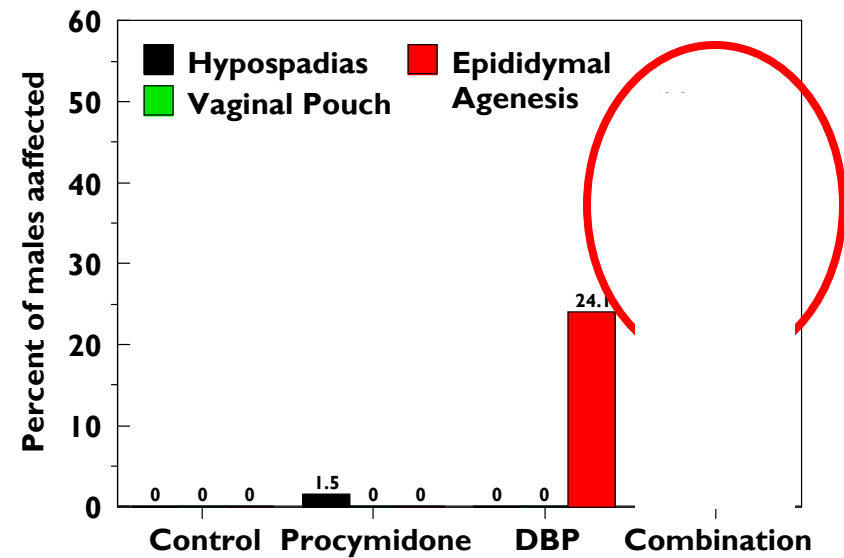
Binary mixture studies with a phthalate plus a pesticide

Linuron plus BBP mixture study. A pesticide that is an AR antagonist and inhibits testosterone synthesis plus a phthalate (Hotchkiss et al. 2004)



Hypospadias: 0+0=56%

AR Antagonist plus Phthalate mixture study (Hotchkiss et al, 2010)



Hypospadias: 1.5+0=49%

Disrupting the AR Pathway by Multiple mechanisms of toxicity.

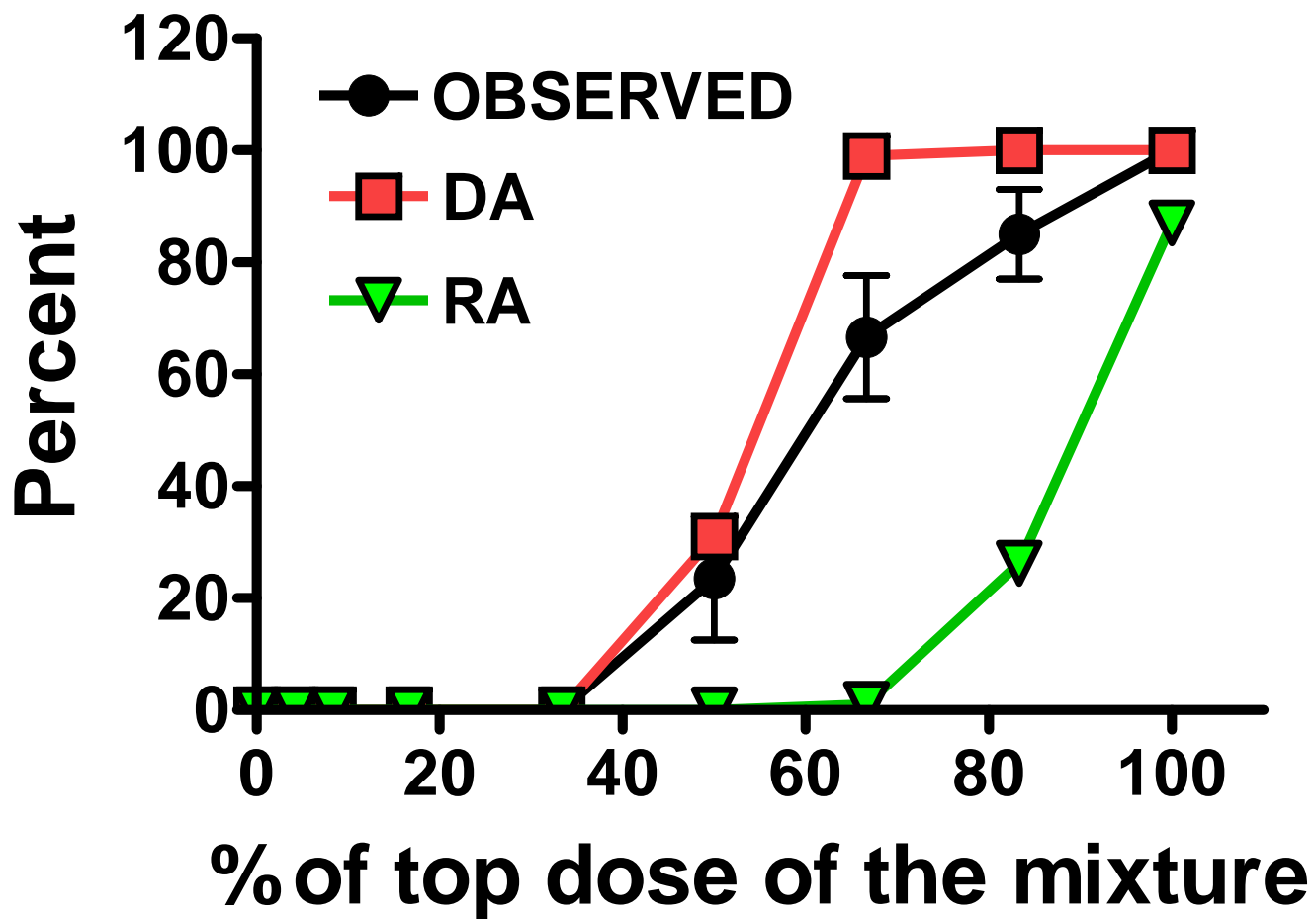
IN UTERO EXPOSURE TO PROCYMIDONE AND DIBUTYL PHTHALATE

Hotchkiss et al, Reproductive Toxicology 2010.

- **Exposure from gestational day 14-18 with the mixture at 100, 83, 67, 50, 33, 17, 8, 4, or 0% of the top dose.**
- **The top dose contained PRO at 150 mg/kg/d and DBP at 1125 mg/kg/d and was expected to induce 100% incidence of malformations.**

PROCYMIDONE AND DIBUTYL PHTHALATE

Hypospadias

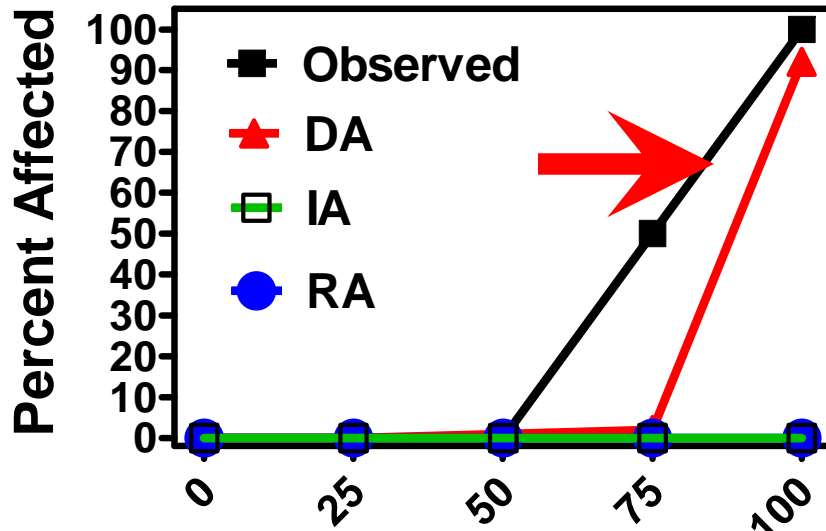


“MegaMix1” Study: 7 antiandrogens

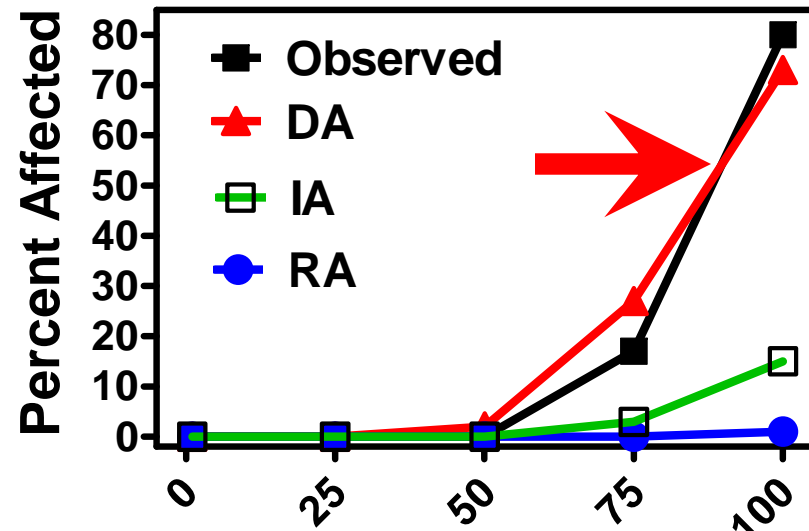
Rider et al. 2008. Int J Androl

- Pregnant rats were dosed from GD 14-18 and male offspring were examined assessed for effects through adult life.
- The “high dose” group, termed the ED₁₀₀, included the 7 chemicals, each at 1/7th their ED₁₀₀ for malformations
- **Doses: ED₁₀₀ and 75%, 50% and 25% of the ED₁₀₀**
- Top dose: vinclozolin 15, procymidone 15, prochloraz 35, linuron 20, and BBP, DBP and DEHP at 150 mg/kg/d per phthalate

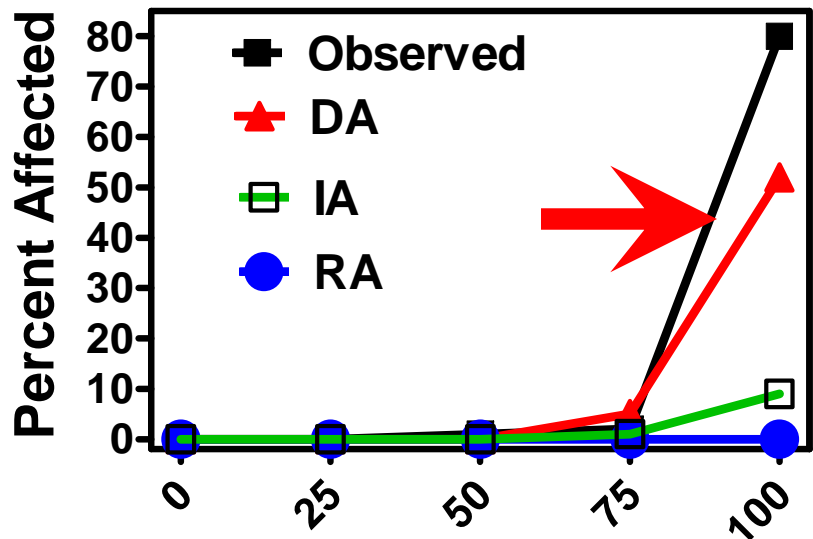
Hypospadias



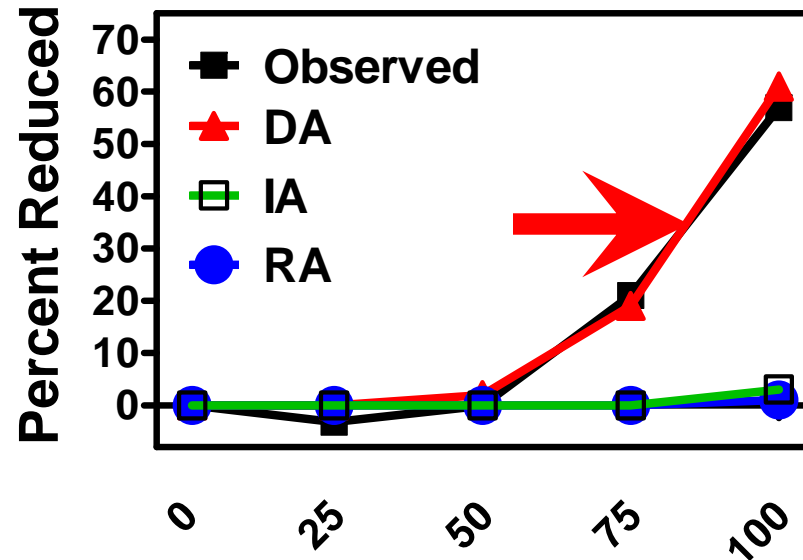
Epididymal Agenesis



Undescended Testes



Seminal vesicle weight



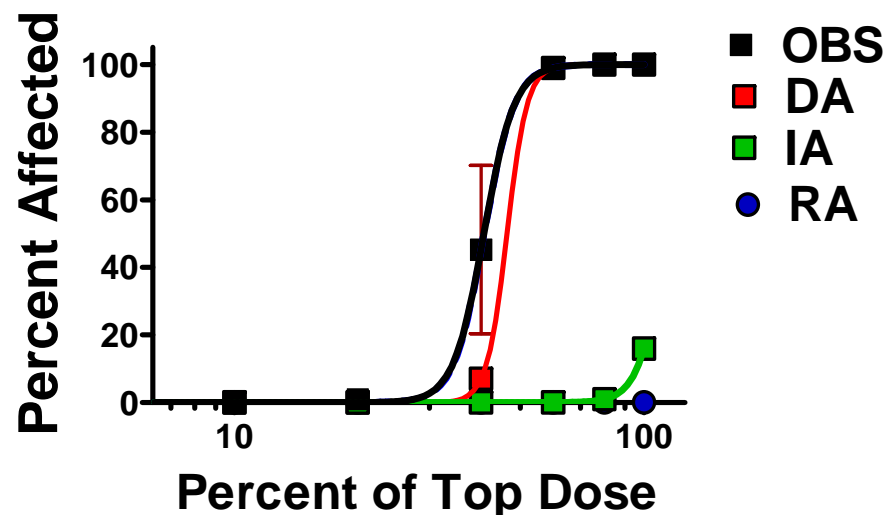
Percent of Top Dose of the Mixture

Values are no. affected/total no. Not litter means

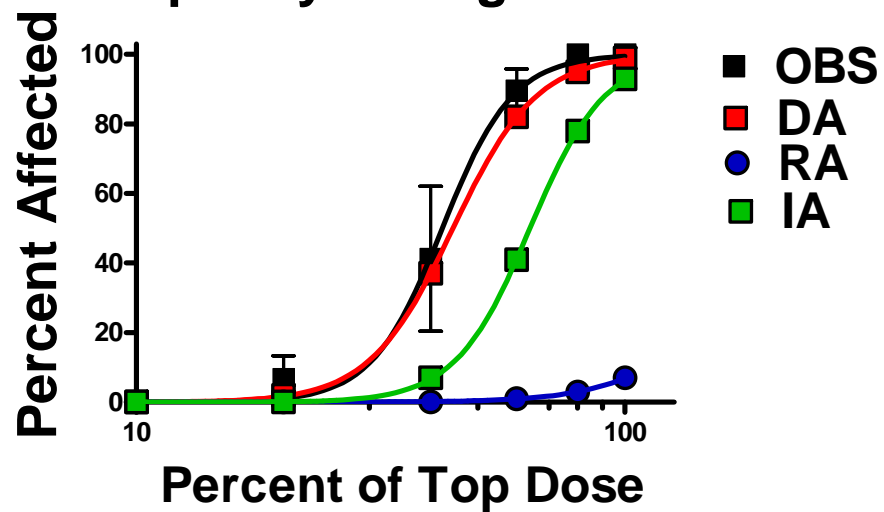
by Cynthia

Malformations in F1 male rats: Megamix 2

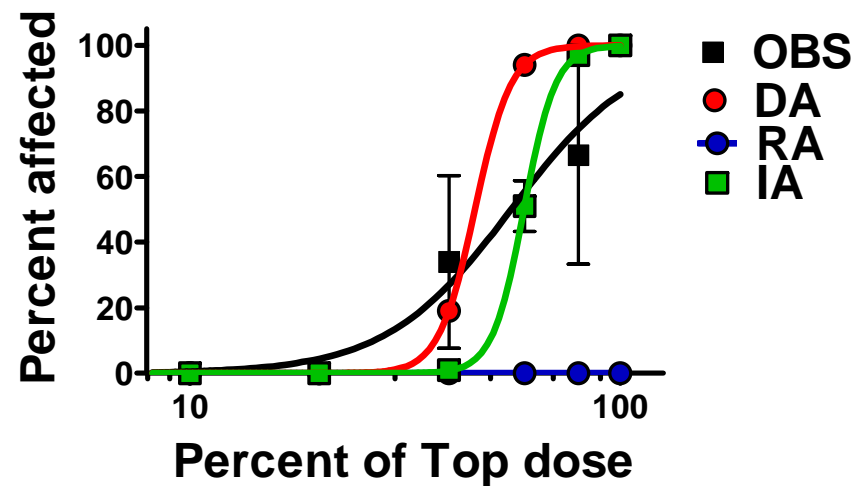
Hypospadias Obs vs predicted



Epididymal Agenesis



Undescended testes



Summary of results on the AR Pathway

- Dose Addition is the most logical model for the data
- Response addition does not explain the results
- DA is consistent with the biology of hormone action
- Phthalates, vinclozolin, procymidone, linuron and prochloraz all act on the fetal tissues by disrupting a “Common Pathway”
- What the tissues “see” is a reduction in AR bound to an androgen so in both cases androgen-dependent gene expression is attenuated
 - AR antagonists do this by preventing T from binding AR
 - T synthesis inhibitors do this by reducing T levels
 - The disrupted developing tissue does not distinguish among these two events.

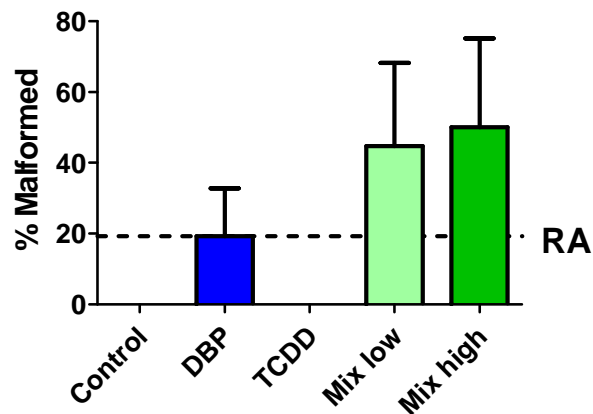
Disrupting Multiple Pathways in common tissues:

Altered sexual differentiation through disruption of AhR and AR signaling pathways

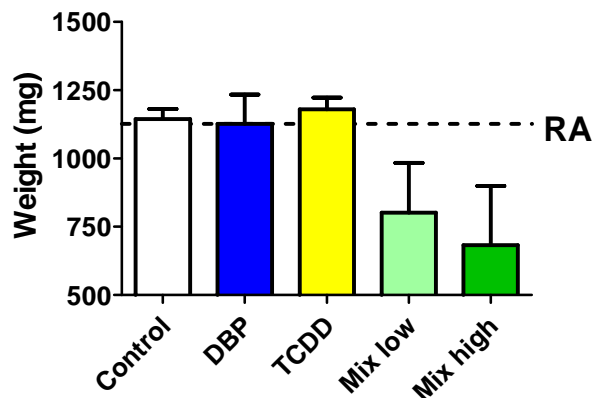
- Dibutyl phthalate (DBP) disrupts male development by decreasing testosterone production by the testes and decreasing expression of insl3 (a protein involved in testicular descent).
- 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) disrupts male reproductive tract development through an unknown mechanism of action that apparently does not involve the androgen signaling pathway.

DBP+TCDD malformations and organ weight reductions that exceeded Response Addition predictions

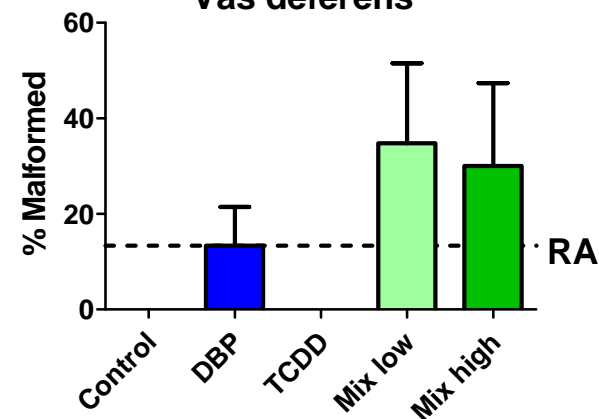
Epididymal and Testicular Malformations



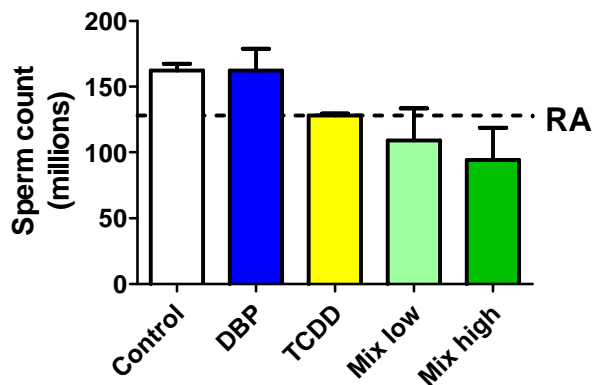
Epididymal Weight



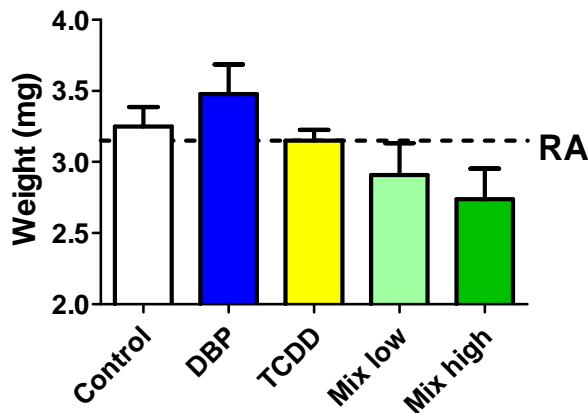
Agnesis of the Vas deferens



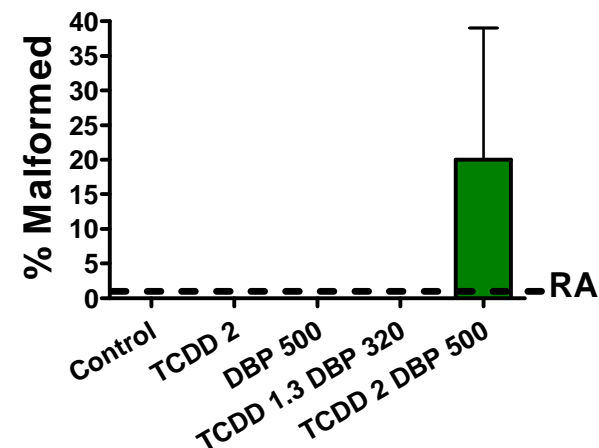
Epididymal Sperm Numbers



Paired Testis Weight

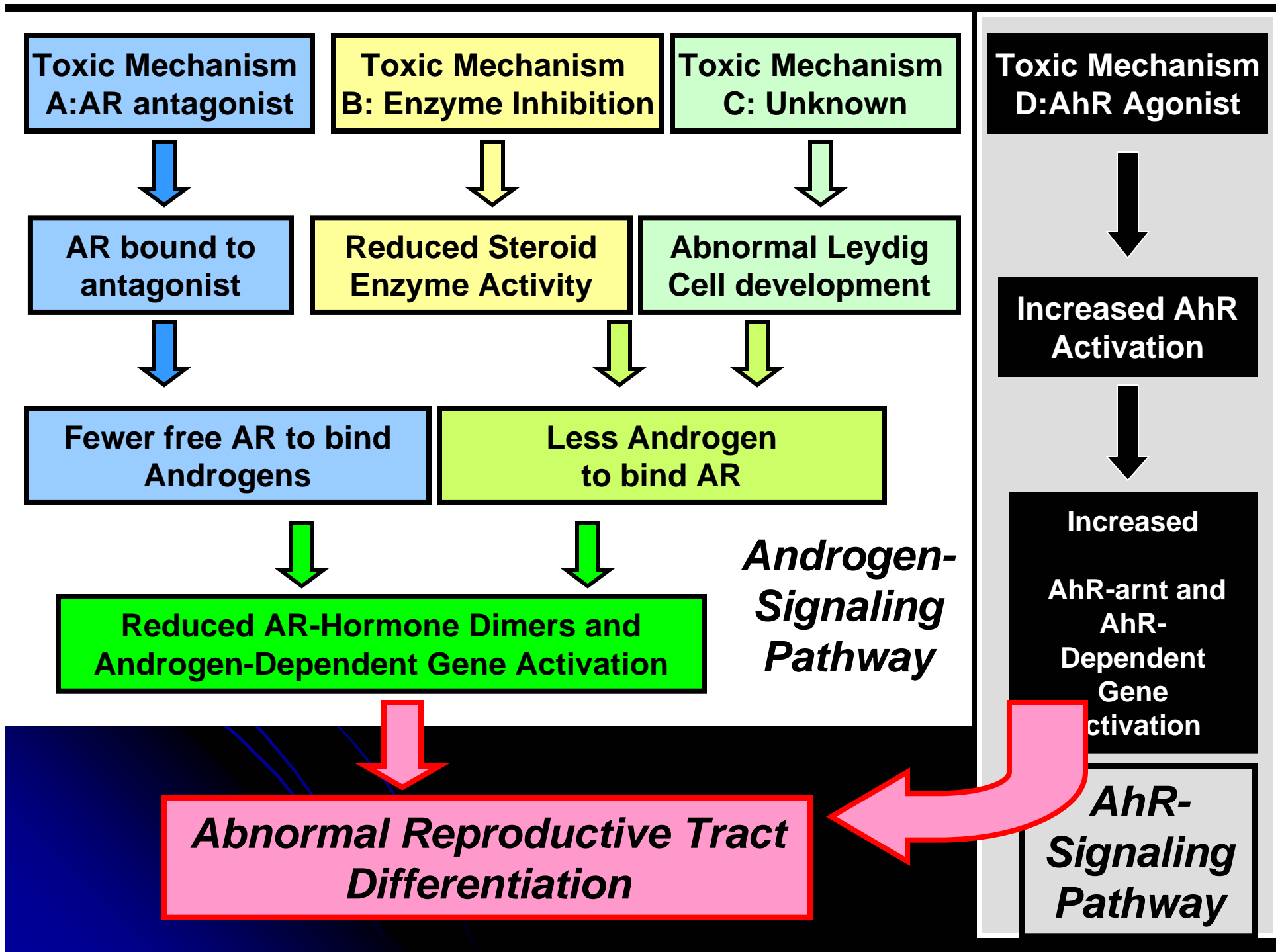


Malformed External genitalia



Cumulative risk assessment using a Framework based upon Disruption of a Common System/developing tissue

- **Cumulative Risk assessments would be conducted on all the chemicals that disrupt common reproductive tissues**
- **Differentiation of androgen-dependent tissues depends upon critical interactions of dynamic**
 - **inter-connected pathways.**
- **All the chemicals that affect the same tissue would be considered in a single cumulative risk assessment and the effects of a mixture would be predicted using the relative potencies on a tissue-by-tissue basis.**



Fetal Phthalate Screening

Hazard ID for Risk Assessment

- ***In vitro* assays and High Through-Put Screening are Negative or fail to provide relevant signals**
- PEs are **NOT** AR antagonists
- PEs alter fetal testis development, reducing androgen and insl3-hormones
- ***FPS is an in vivo short term screening test to detect chemicals that disrupt fetal testosterone synthesis and testis gene expression***
 - *In utero* exposure GD 14-18 (maternal oral dosing)
 - Single high dose phthalate
 - Ex vivo fetal testicular testosterone production
 - **Fetal testis gene expression**
 - follow-up with dose response for “positives”
- ***FPS reduction² in T production are predictive of Phthalate Syndrome in F1. What is the Point of Departure?***

Causally Linking Upstream Events to **Downstream**
Adverse effects for risk assessment
"Toxicity Pathway Analysis"

Upstream

Downstream

Fetal Testosterone levels are reduced

•P450 enzyme levels are reduced

P450 and other LC genes
are inhibited

Tissues are
smaller at
lower doses
with less T

Tissues are
malformed
at high
doses

Phthalates alter Leydig Cell
migration, differentiation

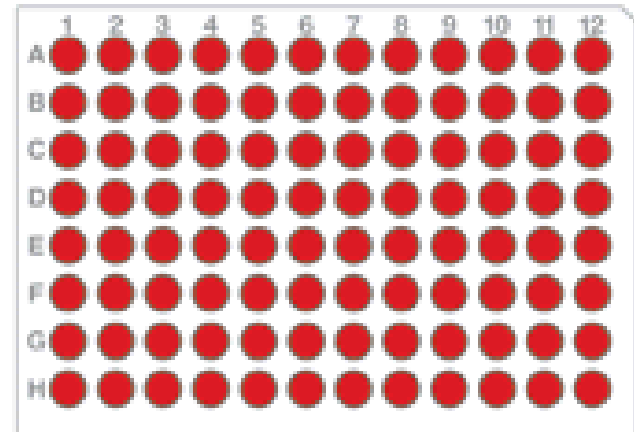
Initiating Event: Unknown is it
relevant to humans?

Cellular proliferation and
differentiation
are blocked in androgen
dependent tissues

Fetal testicular gene expression analysis

- Designed 96-well plate PCR array with individual target genes to assess expression changes following *in utero* phthalate exposure

96 Genes
1 Sample
per Plate



Array gene selection

Sex determination
Testis differentiation
Sertoli cell differentiation
gubernaculum development
Steroidogenesis
AR coregulator proteins
Frizzled signaling pathway
Wnt signaling pathway
PPAR/RXR signaling
neurotropins
Inhibins/Activins
Other nuclear receptors



• *Snoopy Project*

• Interrogate new pathways using 96 gene arrays for 20 new signaling pathways – “snooping” without a hypothesis

• Pathways interrogated to date

- Insulin signaling
- Embryonic stem cell
- Hedgehog
- Osteogenesis
- Hox
- Nuclear receptor
- VegF
- WNT
- JAK STAT
- Rat Growth

Phthalates as developmental reproductive toxicants in the rat

- Female rat – pregnancy loss
- Female Primate – uterine and ovarian histopathology
- Male rats and other mammals – Testicular Toxicants
- In utero alterations of sexual differentiation
 - Male
 - Rats: PEs reduce fetal rat Leydig cell testosterone and insulin-like 3 (insl3) hormone levels. the phthalate syndrome
 - Rabbits – reduced sperm counts
 - Many mammalian species - multinucleated germ cells
 - Female
 - Reproductive tract malformations and reduced fecundity in F1 female rats

Diverse Phylogenetic relationships of species in the class Mammalia that are vulnerable to phthalate-induced developmental or reproductive toxicity.



?

ORDER PRIMATES
Family Hominidae

Eutherian Mammals

Subclass

+

Carnivora
Ferret

+

Lagomorpha
Rabbit

Rodentia

Order



+

Suborder
Hystricognathi
Guinea Pig



Suborder
Sciurognathi
Family Muridae

+

Subfamily
Murinae
Rats and mice



+/-

Subfamily
Cricetinae
Hamsters



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