

Effects of mixtures of phthalates and other toxicants on sexual differentiation in rats:  
*A risk assessment framework based upon disruption of common developing systems*



*USEPA scientist  
grapples with difficult  
environmental issues*

*L. Earl Gray Jr.*

*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**

# Developmental Reproductive Toxicants

## 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
- Prochloraz
- p,p' DDE and other o,p'- and p,p' DDT metabolites

## Inhibitors of fetal androgen synthesis

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

- DEHP
- DPP, DCHP
- BBP, DBP, DiBP
- DiHP, DHP, DHeP
- DINP
- Linuron
- Prochloraz

## Estrogens

Methoxychlor  
Ethinyl Estradiol  
Bisphenol A

## Fetal Germ Cell Toxicants

Busulfan  
Diazo dyes

## Steroidogenesis inhibitors

Prochloraz  
Linuron  
Ketoconazole  
Fenarimol

## Androgens

Testosterone  
Trenbolone

## Dioxins and PCBs

Dioxin

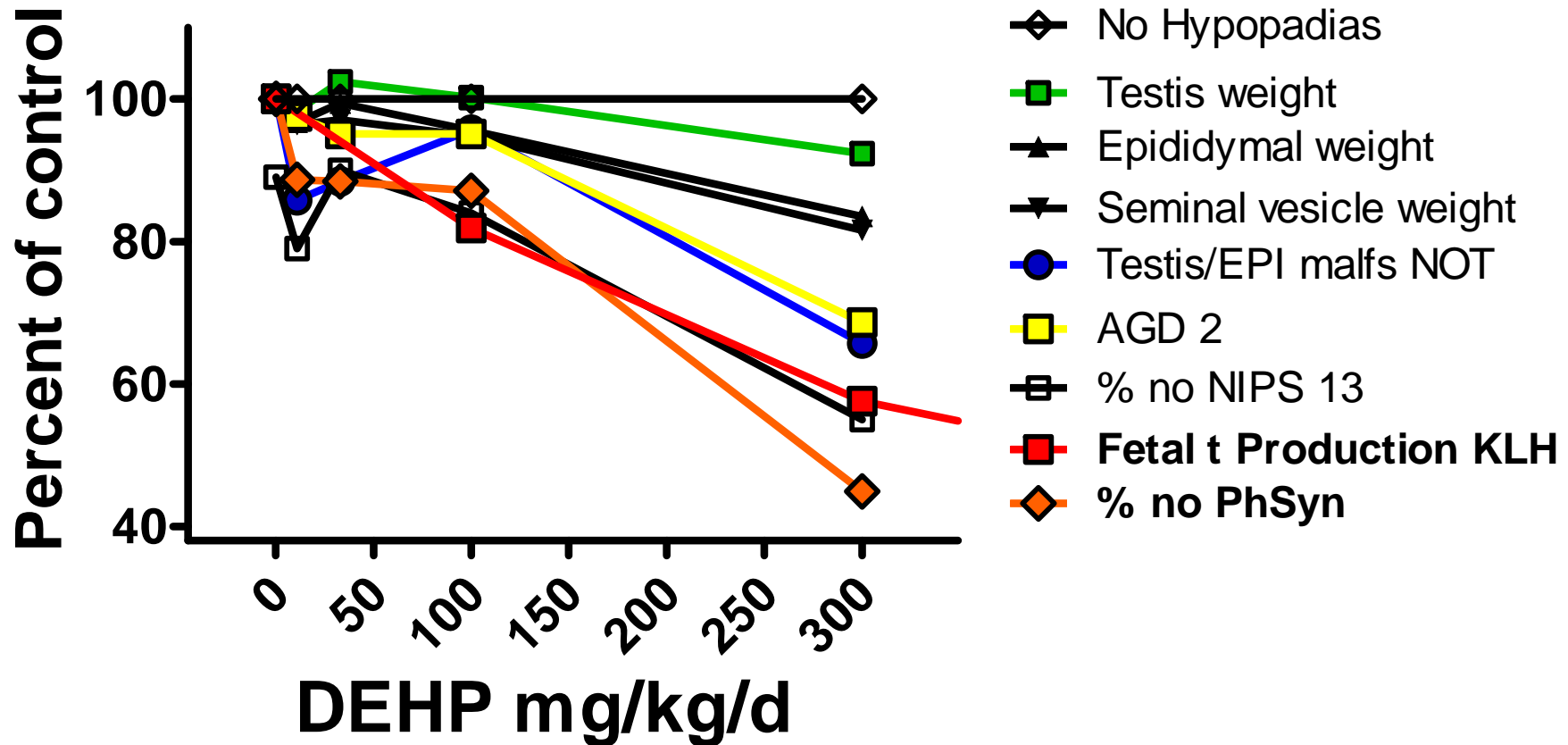
PCB 169 congener

**•In Utero exposure and measure Hormone dependent endpoints male rat offspring as adults**

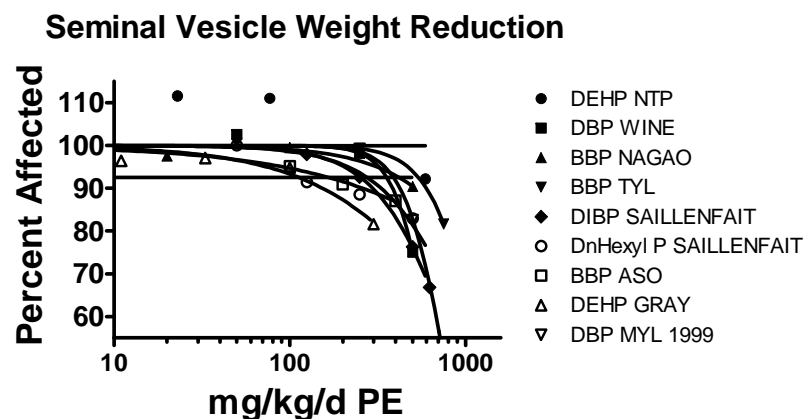
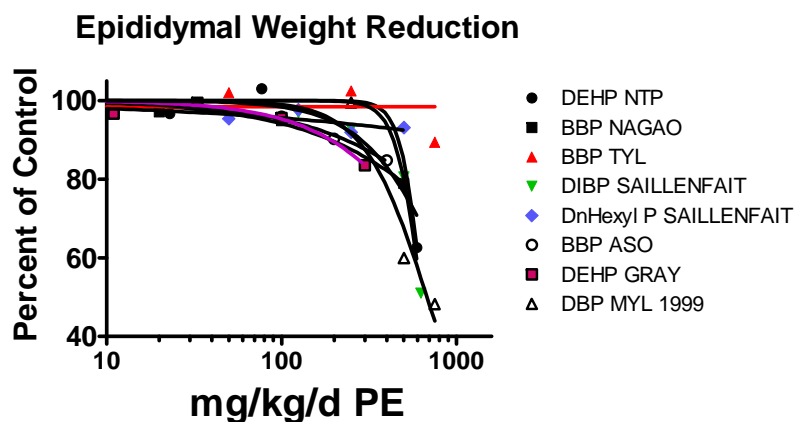
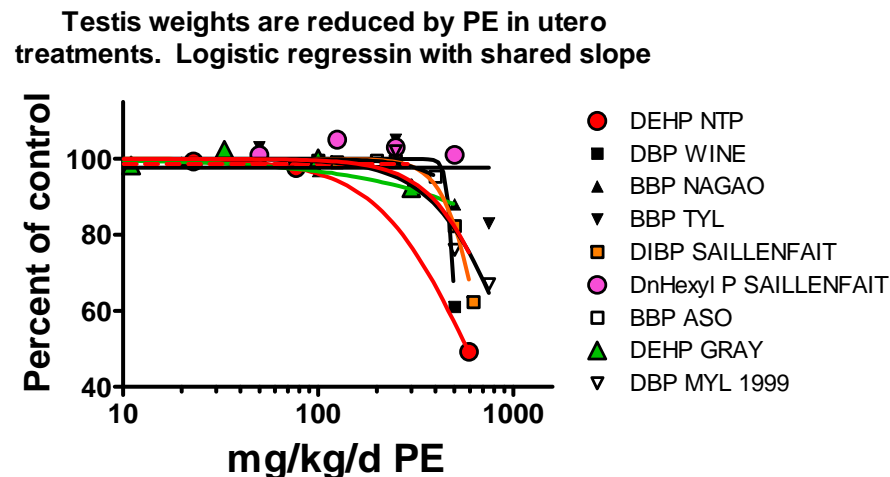
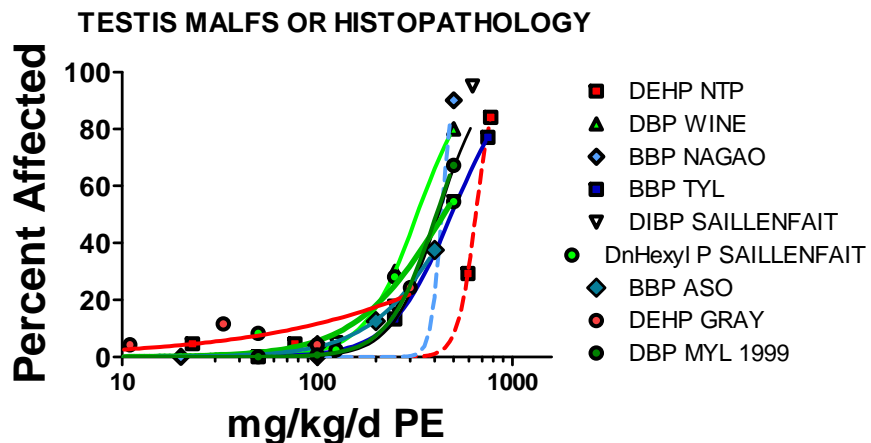
- Anogenital distance at birth
- Nipple/ areolar numbers in infants
- Reproductive Malformations
  - Undescended testes
  - Gubernacular abnormalities
  - Epididymal agenesis
  - Ventral prostate agenesis
  - Seminal vesicle agenesis
  - Vas deferens agenesis
  - Nipples
  - Hypospadias
  - Vaginal pouch
- Reproductive Organ Weights
  - Glans penis
  - Ventral prostate
  - Seminal vesicle
  - Testes
  - Epididymides
  - Levator ani bulbocavernosus
  - Cowper's glands
- Testis and epididymal histopathology

F1 male rat offspring: Reproductive endpoints sensitive to lower dosage levels of phthalates in utero. Critical “adverse effects”: % with Phthalate Syndrome and Fetal androgen production.

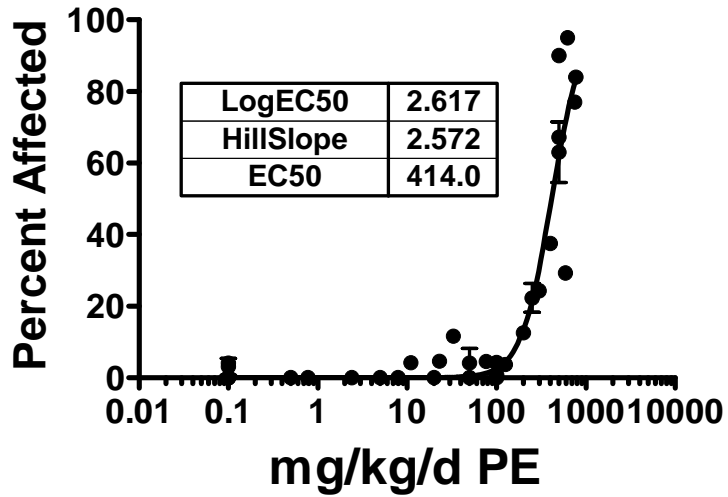
DEHP DR Gray et al 2009



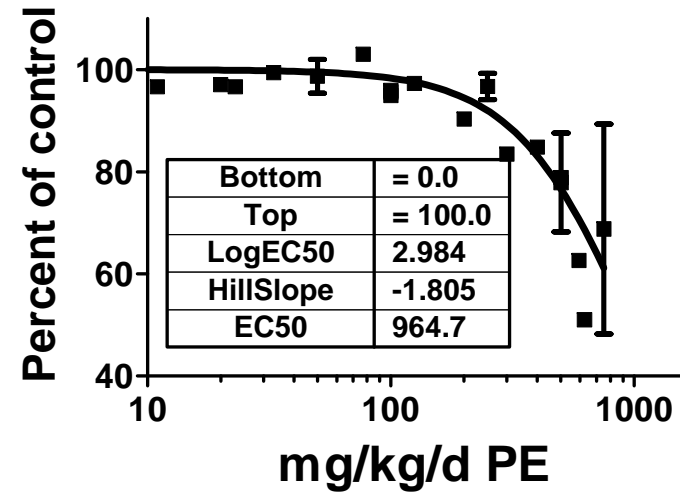
# PE Dose Response reproductive effects on F1 Male rat offspring indicate that DEHP, DBP, DiBP, BBP and Dihexyl phthalate are equipotent. DPP is more potent and DINP is less potent than this group of PEs



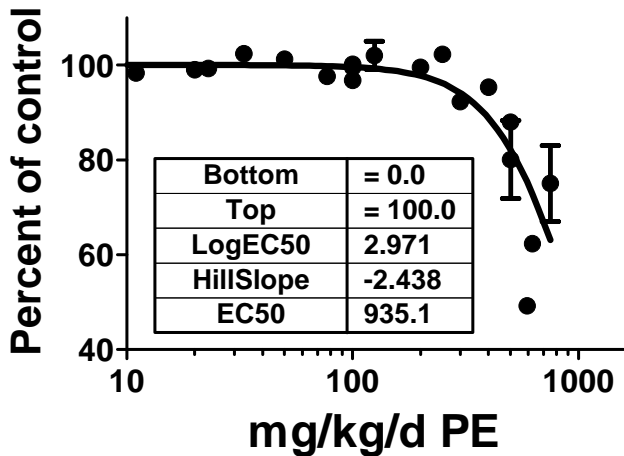
Testis/EPI malfs data pooled studies



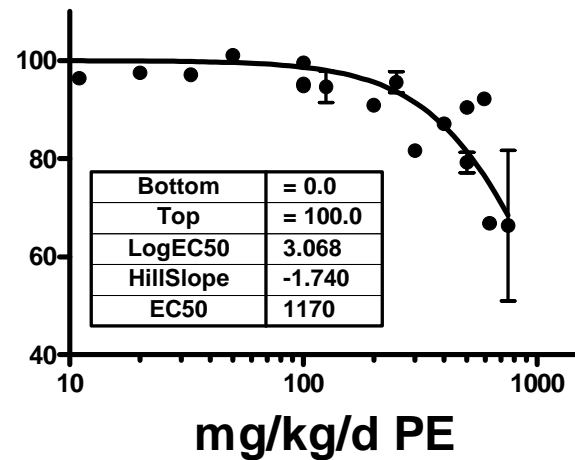
Adult male F1 epididymal weight data pooled from several rat studies



Adult male F1 rat testis weight data pooled from several rat studies



Adult male F1 seminal weight data pooled from several rat studies



**Fetal endocrine changes demonstrate the relative potency (RP) of individual PEs is consistent with their RP to induce reproductive tract alterations seen in postnatal life. Thus, changes in fetal androgen levels can be used to “screen” phthalates for their potential to induce malformations and possibly as a “critical effect” in individual and cumulative risk assessments.**

- Fetal Phthalate screen (FPS)
  - Dose dam with PE at a single high dosage group
  - Gestational exposure during critical period of sexual differentiation
  - On Gestational Day 18 necropsy dam
    - Measure testis fetal testis Testosterone (T) production
    - Testis gene expression
      - STAR
      - Insl3
      - Cyp11a
  - For “Positives”
    - Run dose response studies
    - Use potency factors to execute mixture studies
    - For “important” PEs with data gaps run a postnatal study

# FPS Results to date

## ● Positives

- DINP – both CAS #s \*
- Diheptyl phthalate
- Diisoheptyl phthalate
- Dihexyl phthalate
- Dibutyl phthalate \*
- Diisobutyl phthalate \*
- Benzylbutyl phthalate
- Dicyclohexyl phthalate
- Dipentyl phthalate \*

## ● Negatives

- Diethyl phthalate
- Dioctyl terephthalate
- Hexamoll DINCH
- Di-2-ethylhexyl tetrabromo phthalate

## ● Future studies

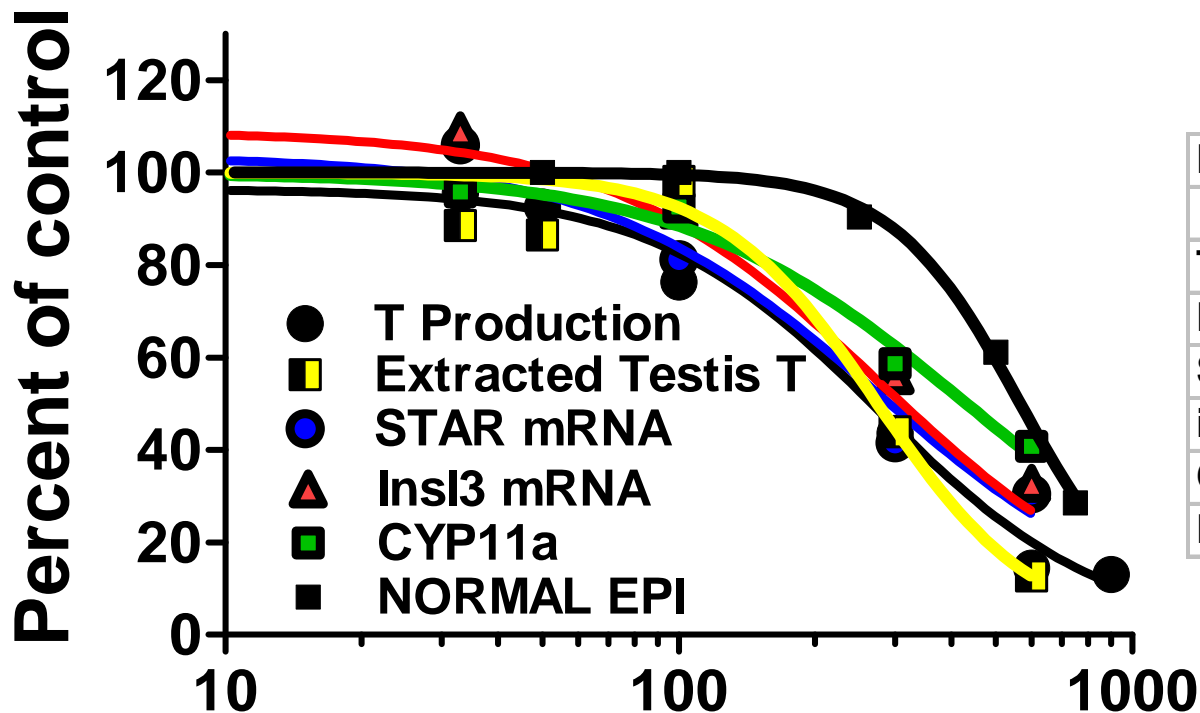
- DMP, Dipropyl P, DIDP, DPHP, others

\* FPS DOSE RESPONSE STUDIES ONGOING



Fetal T production is more sensitive to Phthalate ester (PE) disruption than are testis genes of postnatal effects (epididymal malformations) and T prod is less variable resulting in lower NOELs and BMDs. Below is a generic example.

## PE IN UTERO EFFECTS ON FETAL AND POSTNATAL MALE RATS



Endpoint	ED50
T Prod	231
Extracted Testis T	275
STAR mRNA	280
insl3 mRNA	372
CYP 11a	431
Epididymal agensis	567

# *In utero* mixture studies

## Summary

- *Similar cellular and molecular mechanisms of action*
  - Binary mixtures – pairs of chemicals
  - Mixture of 5 phthalates
- **Diverse cellular and molecular mechanisms of action**
  - Binary mixtures – pairs of chemicals
  - Mixture of 7 chemicals including pesticides and phthalates
  - Mixture of 10 chemicals

## **Initial Step in a Cumulative Risk Assessment as outlined by the USEPA and other regulator agencies.**

- **Identification of a group of chemicals to be included in a**

### ***Common Mechanism Group***

- **chemicals that induce a common toxic effect by a common mechanism of toxicity.**
- 

# 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 has been:

- **NO**

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

## Response addition (RA)

- $0 + 0 + \dots + 0 = \text{No Adverse Effects}$

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

- $0 + 0 + \dots + 0 = 100\% \text{ Malformations}$

# 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:*

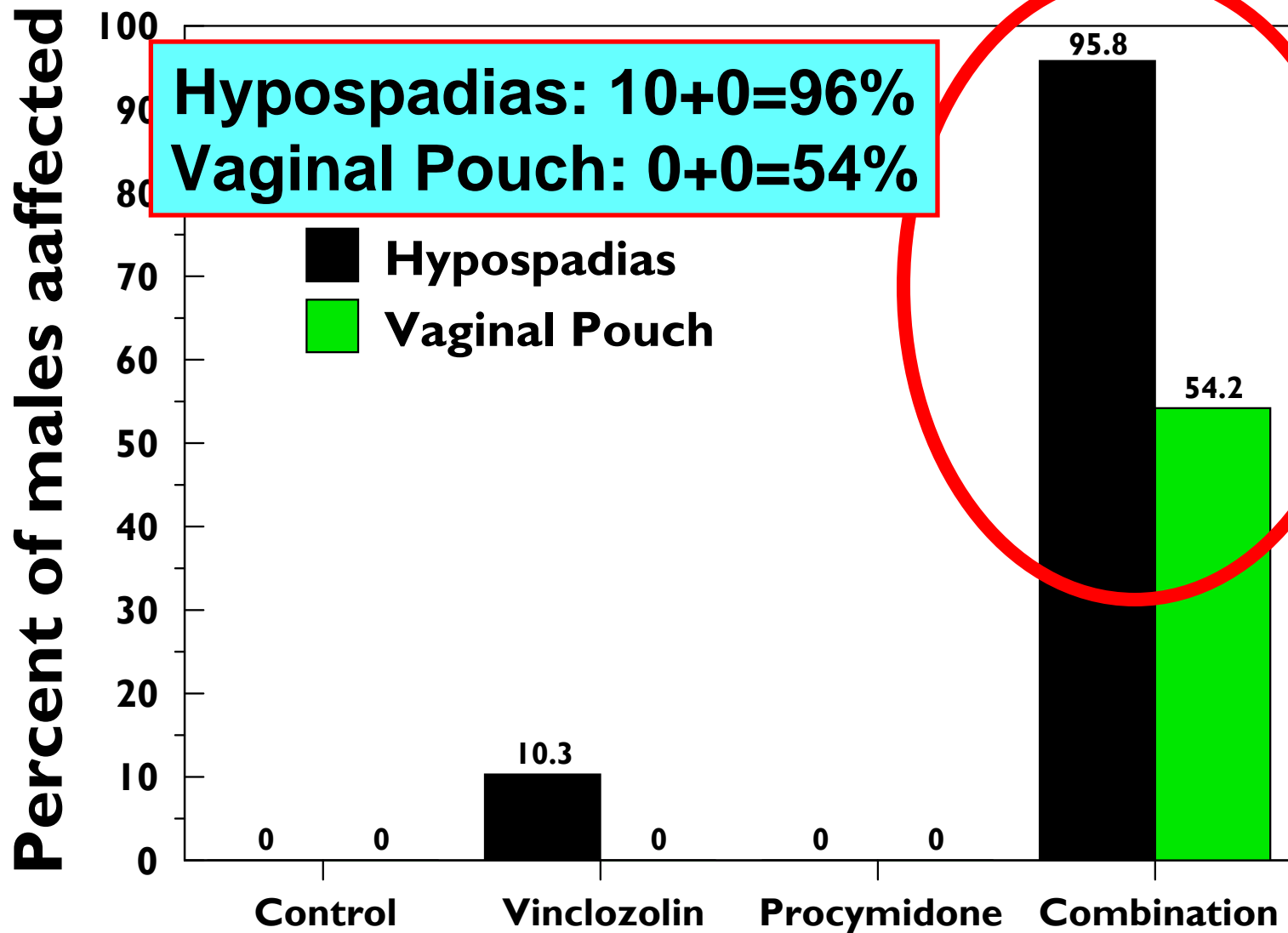
Chemicals that disrupt the development of a common reproductive tissue/system during sexual differentiation will produce dose additive responses, regardless of the molecular mechanism or the signaling pathway that is disrupted

***Cumulative effects:***

***Common mechanisms of toxicity***



# AR antagonist mixture study

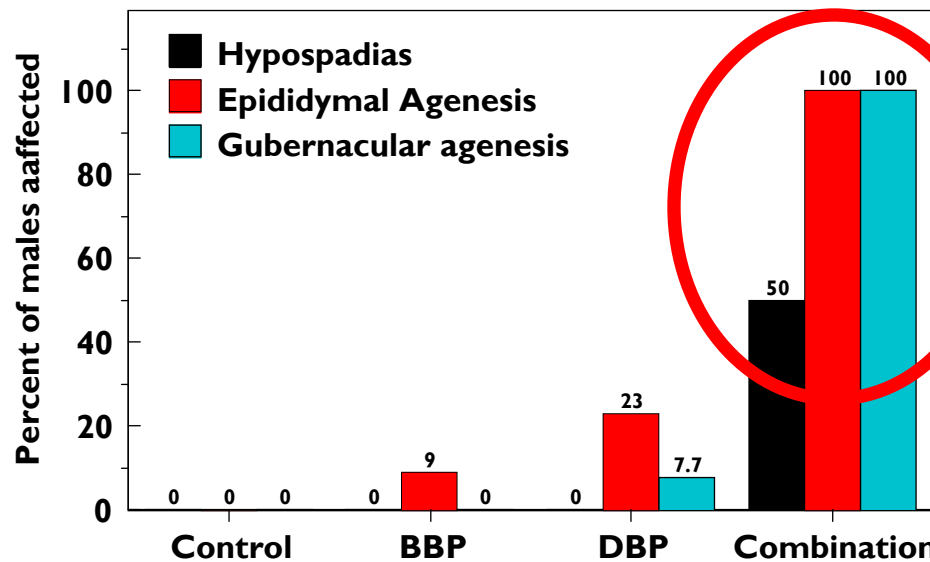


# Cumulative effects of Phthalates

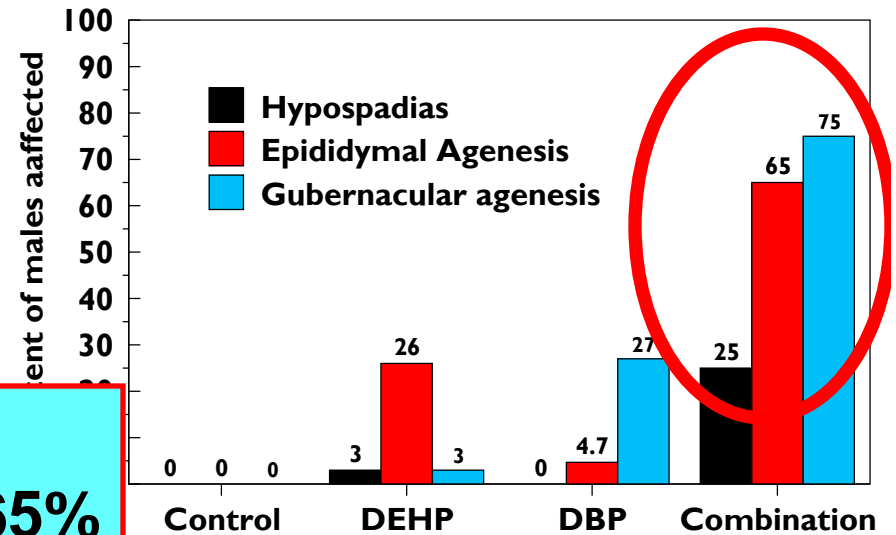
*A Common Mechanism of Toxicity: Altered fetal Leydig cell differentiation and reduced hormone synthesis*

Hypospadias:  $0+0=50\%$

Epididymal agenesis:  $9+23=100\%$



**DBP plus DEHP mixture study**  
**phthalates with no common**  
**metabolite**  
(Howdeshell et al 2007)



Hypospadias:  $3+0=25\%$

Epididymal agenesis:  $26+5=65\%$



# Common mechanism of Toxicity

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

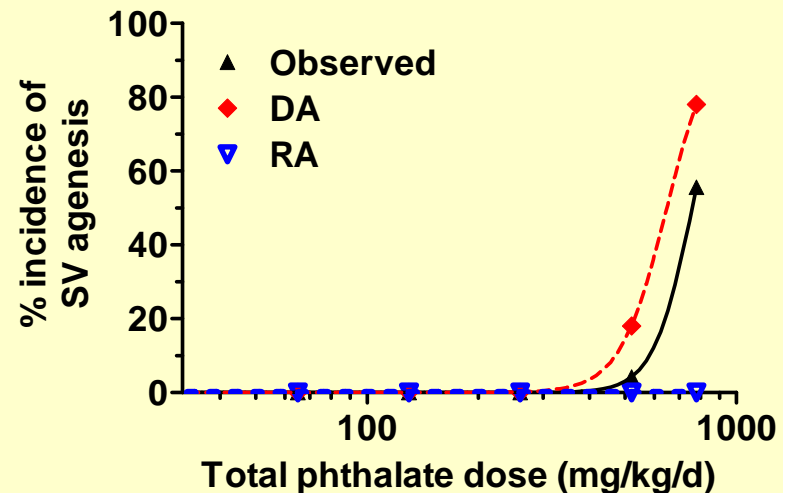
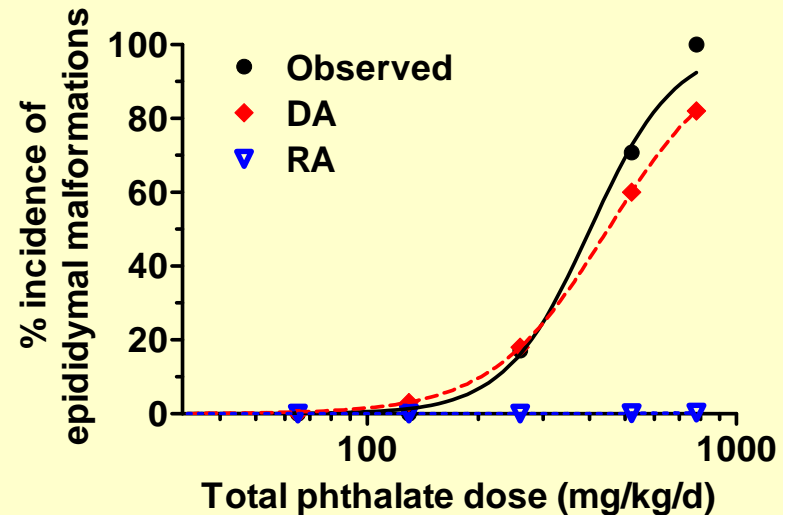
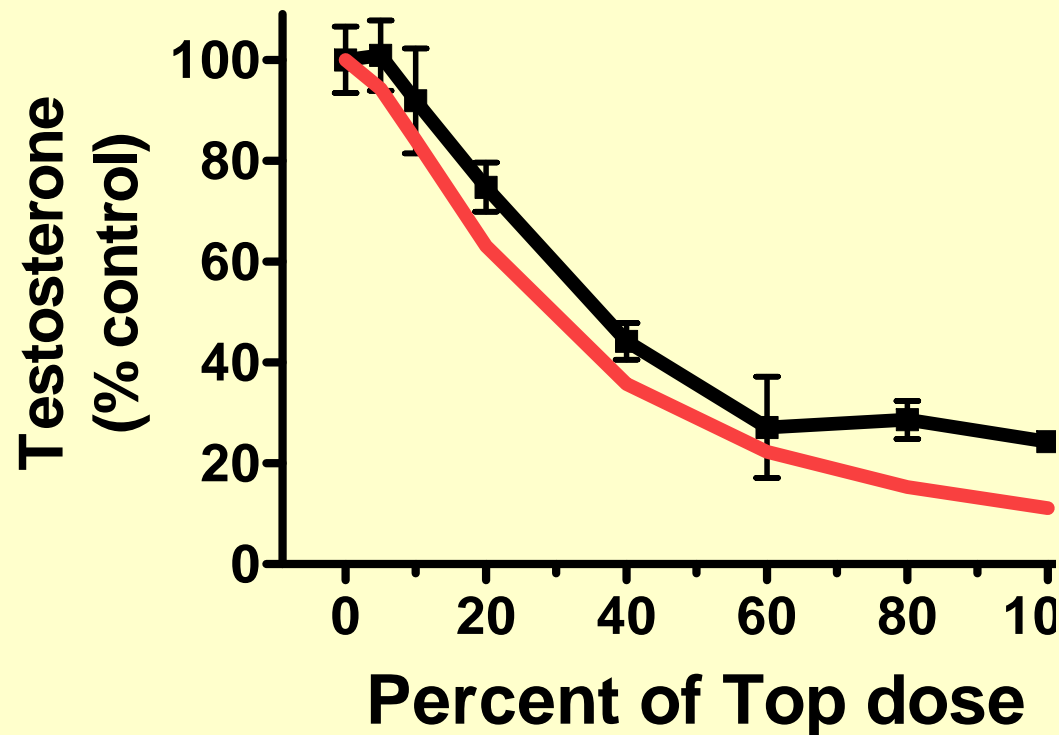
- Individual studies

- DBP, DiBP, BBP, DEHP, DPP administered at several dosage levels on GD 8-18. Fetal testis collected on GD 18 and fetal T production measured.

- Mixture study

- Five phthalates were administered as a mixture 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 effect predicted from dose addition modeling



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

# Unexpected high incidence of reproductive tract malformations in female rat offspring in the 5 phthalate mixture study

**Uterine and vaginal agenesis**

**Shown here –  
uterus  
unicornis with  
agenesis of the  
lower vaginal  
canal and  
vaginal opening**



## ***Cumulative effects:***

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

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

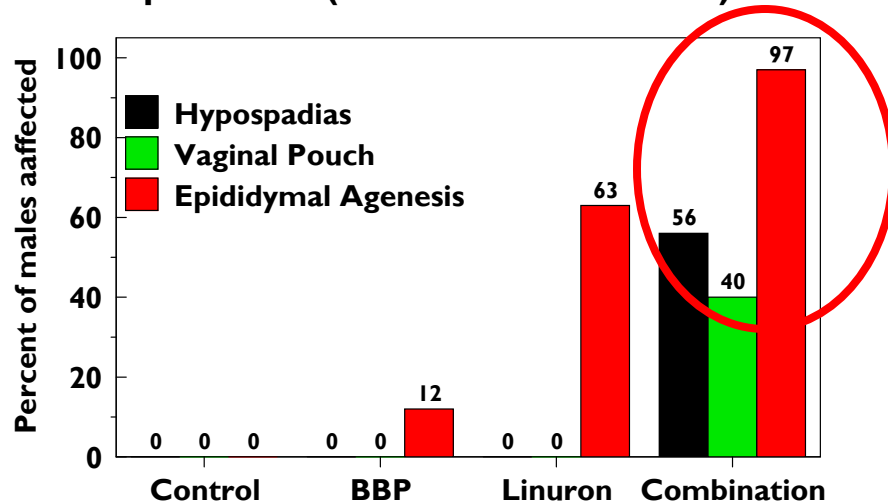


## *Binary mixtures studies with chemicals that act via different mechanisms*

- The chemical pairs include:
  - 1) a phthalate ester plus a herbicide that has dual modes of action (linuron - 75 mg/kg/d and BBP 500 mg/kg/d)(Hotchkiss et al. 2004).
  - 2) a phthalate ester plus an AR antagonist (DBP 500 mg/kg/d and procymidone 50 mg/kg/d)
  - Two studies
- SD rats were dosed on GD 14-18 with chemicals singly or in pairs at dosage levels equivalent to about one half of the effective dose which causes a 50% incidence (ED<sub>50</sub>) of hypospadias and/or epididymal agenesis.

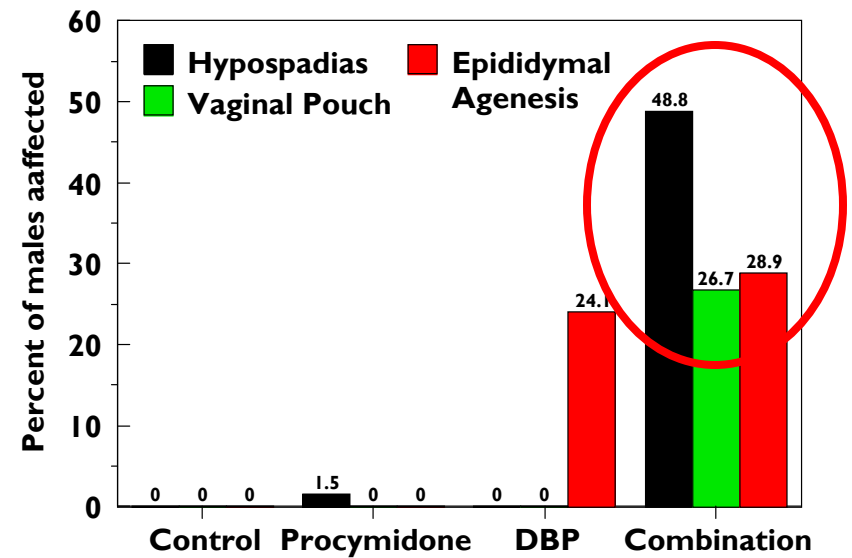
# Binary mixture studies with a phthalate plus a pesticide

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.**

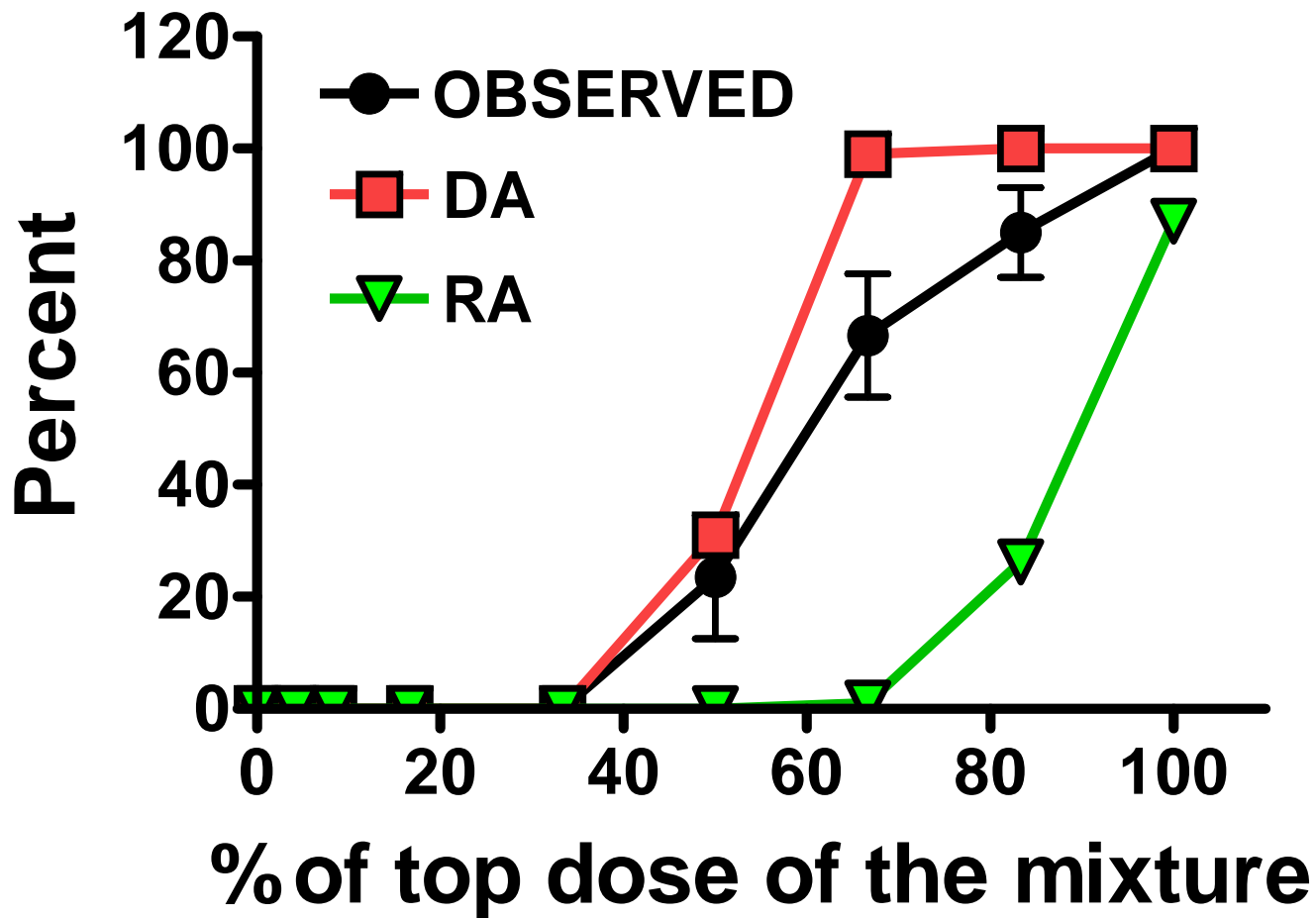
### **IN UTERO EXPOSURE TO THE FUNGICIDE PROCYMIDONE AND DIBUTYL PHTHALATE**

**Hotchkiss et al, Reproductive Toxicology 2010.**

- **Timed-pregnant Sprague-Dawley dams (n=4-10/dosage group) were gavaged daily from gestational day 14-18 with a mixture at 100, 83, 67, 50, 33, 17, 8, 4, or 0% of the top dose.**
- **The top dose of the mixture contained PRO at 150 mg/kg/d and DBP at 1125 mg/kg/d and was expected to induce 100% incidence of malformations.**

# IN UTERO EXPOSURE TO THE FUNGICIDE PROCYMIDONE AND DIBUTYL PHTHALATE PRODUCE DOSE-ADDITIVE DISRUPTIONS OF MALE RAT SEXUAL DIFFERENTIATION

## Hypospadias



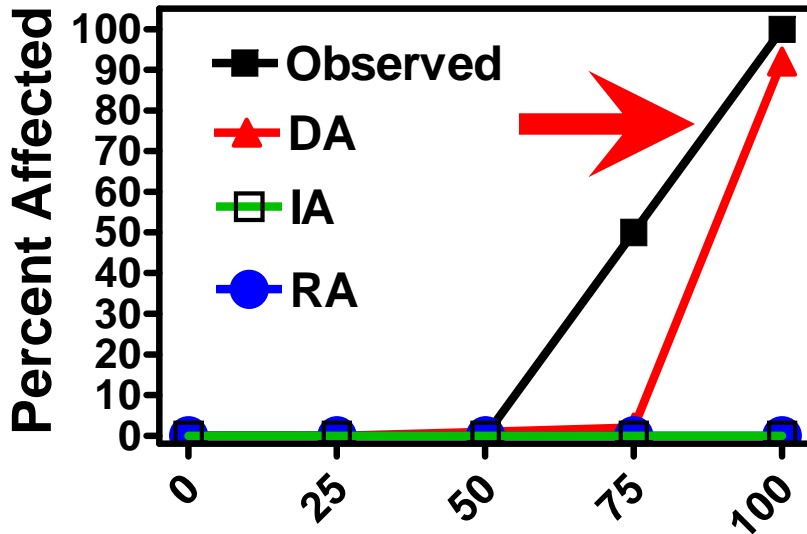


# “MegaMix1” Study: 7 antiandrogens

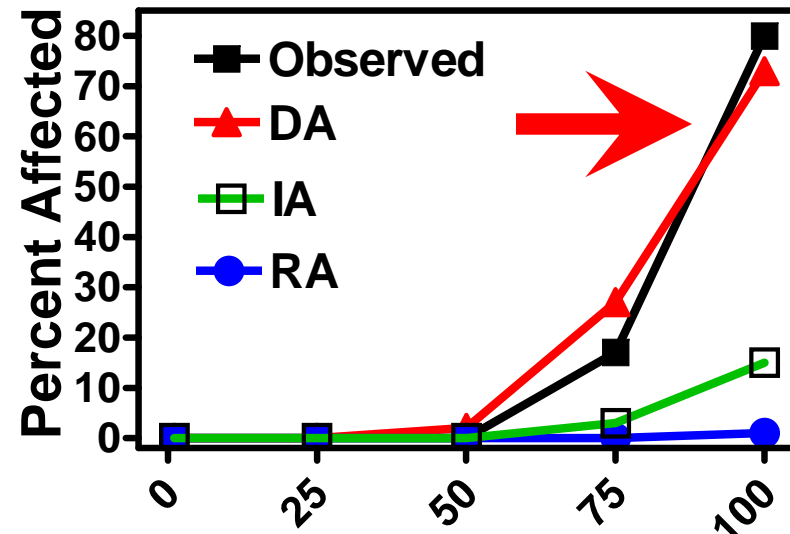
*Rider et al. 2008. Int J Androl*

- 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 ED<sub>100</sub>, included the 7 chemicals, each at 1/7th their ED<sub>100</sub> for malformations
  - vinclozolin 15 mg/kg/d, procymidone 15 mg/kg/d, prochloraz 35 mg/kg/d, linuron 20 mg/kg/d, and BBP, DBP and DEHP at 150 mg/kg/d per phthalate
- Doses: ED<sub>100</sub> and 75%, 50% and 25% of the ED<sub>100</sub>

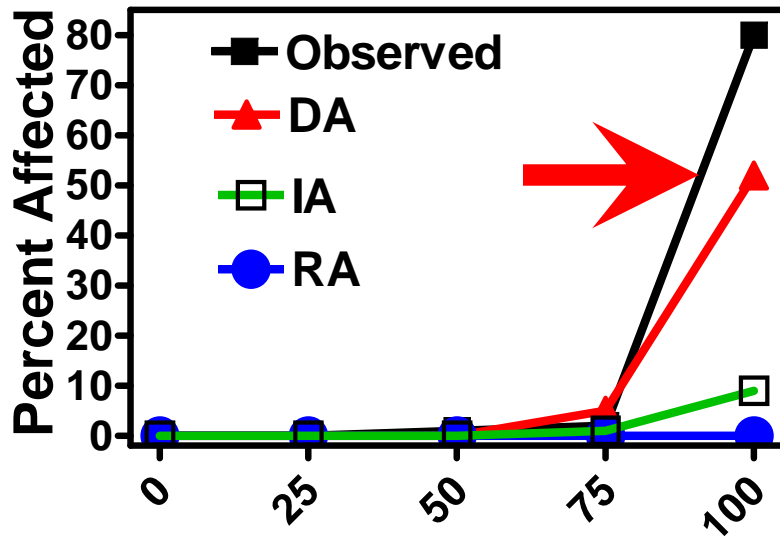
## Hypospadias



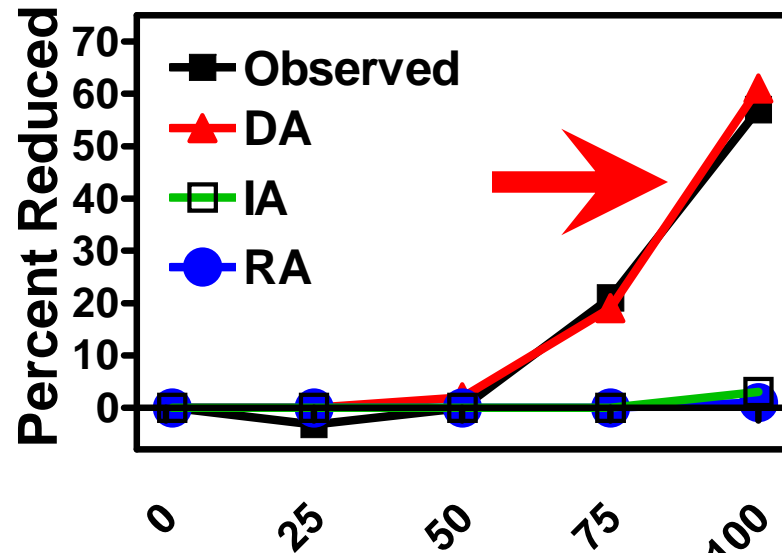
## Epididymal Agenesis



## Undescended Testes



## Seminal vesicle weight



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

by Cynthia

## Disrupting the AR Pathway by Multiple mechanisms of toxicity.

Ten chemical mixture study.

*Rider et al 2010*

Ten “antiandrogenic” chemicals were administered orally to pregnant rats on gestational days 14-18 and the reproductive development of the male offspring was evaluated.

Data were analyzed to determine if the chemicals behaved in a response-, integrated or dose-additive fashion.

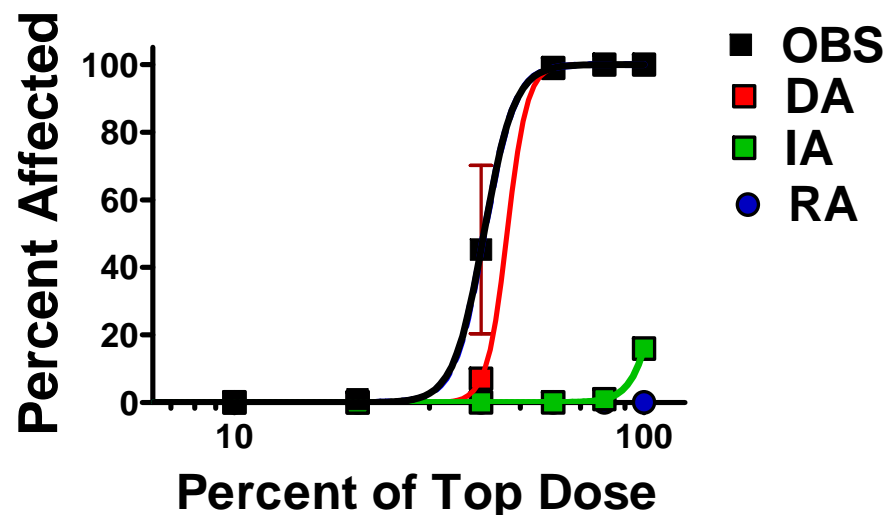
# “MegaMix 2” Study: 10 antiandrogens

- The “high dose” group, termed the ED<sub>100</sub>, included
  - vinclozolin (30 mg/kg/d)
  - procymidone (30)
  - prochloraz (60)
  - linuron (40)
  - Six phthalates: DPP (50) and BBP, DBP, DiBP, DiHP and DEHP (150 per phthalate)

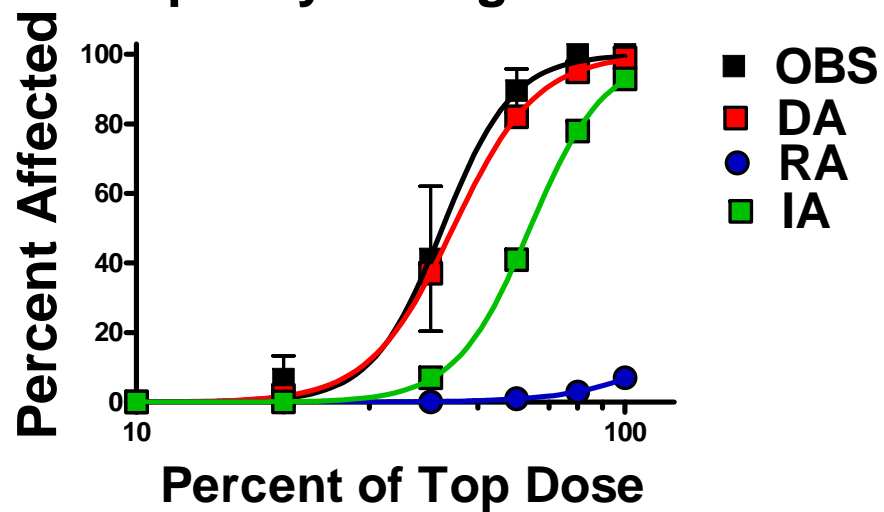
- **Doses: 100%, 80, 60, 40, 20, 10 and 0% of the top dose**

# Malformations in F1 male rats: Megamix 2

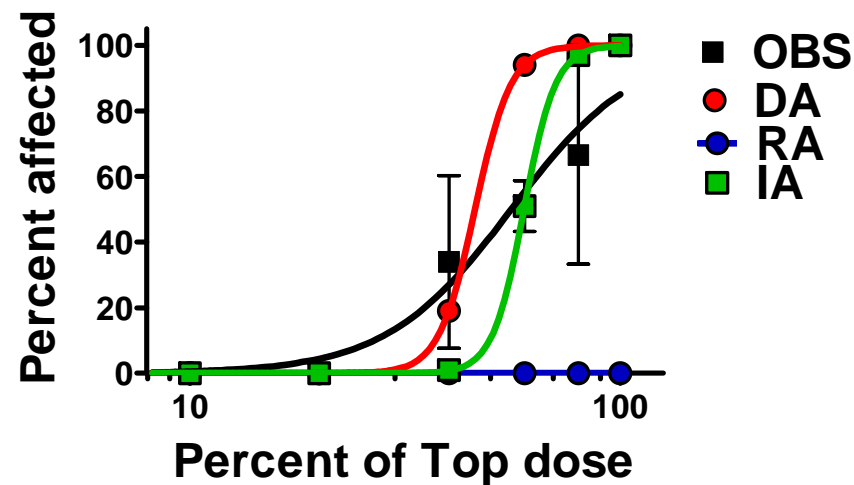
## Hypospadias Obs vs predicted



## Epididymal Agenesis

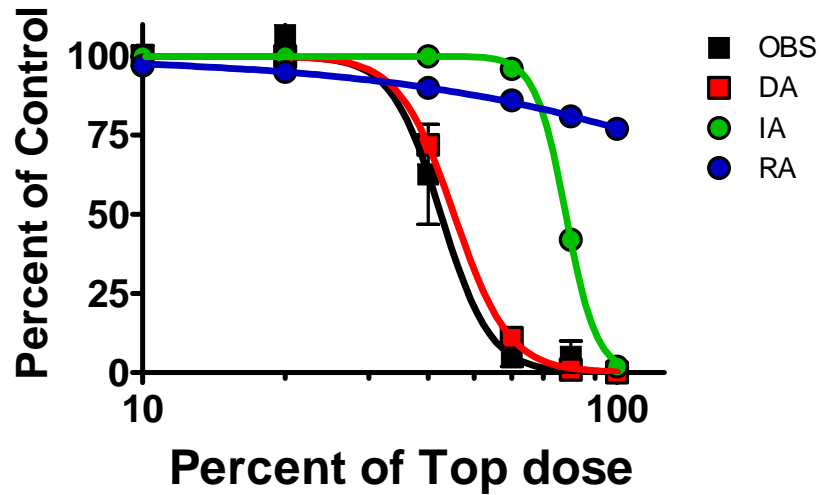


## Undescended testes

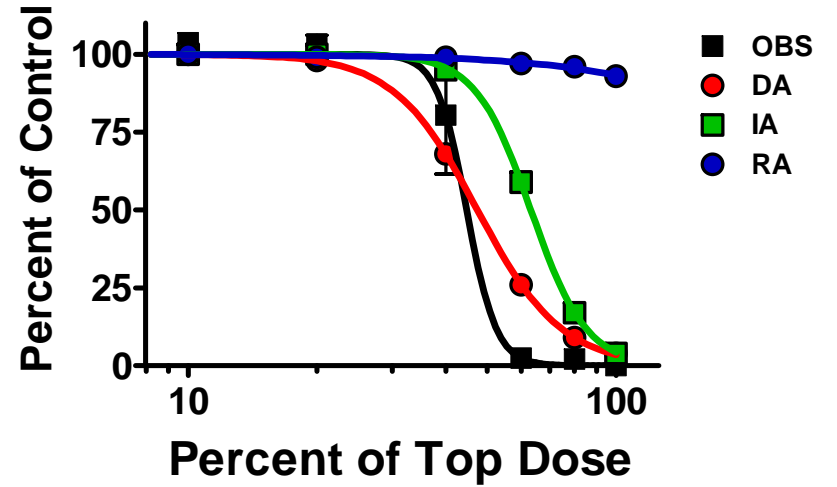


# Organ weights from F1 male rats: Megamix 2 data

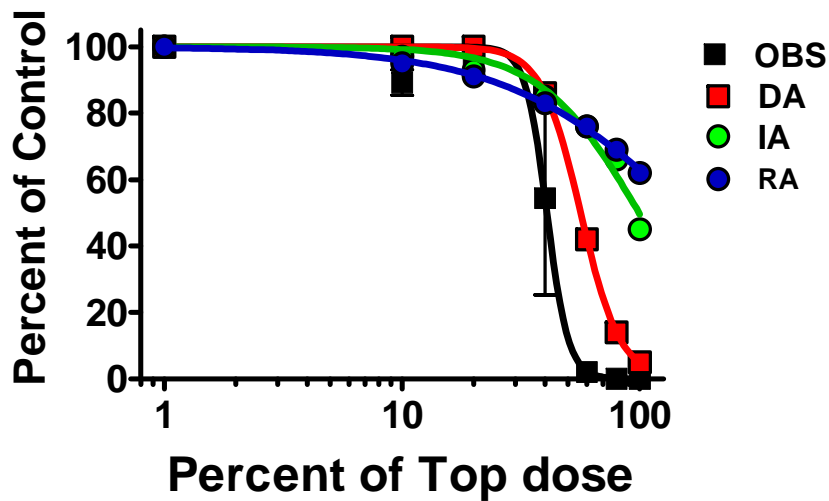
Seminal vesicle wt obs vs predicted



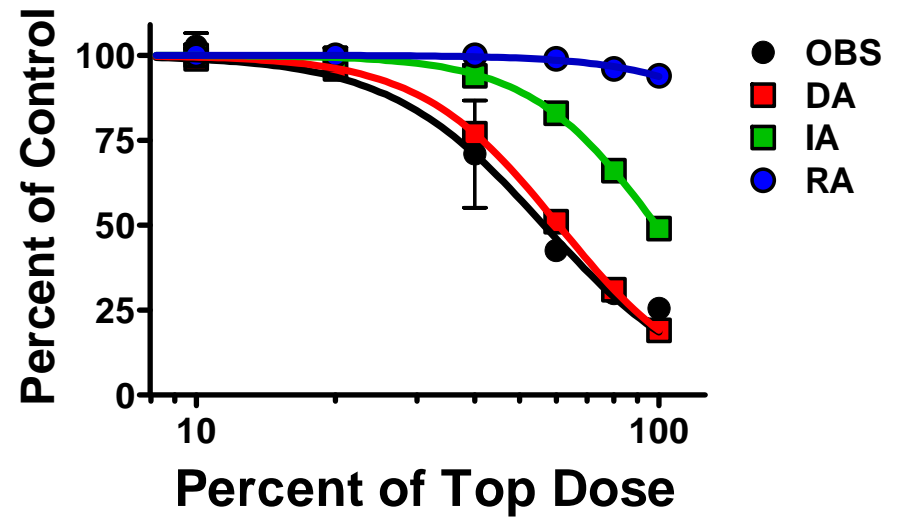
Epididymal WT obs vs predictions



Ventral Prostate weight



LABC wt obs vs predicted



# 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.



## Disrupting Multiple Pathways in common tissues:

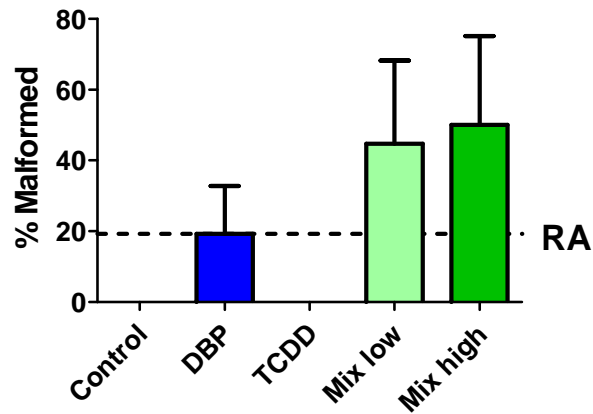
### Altered sexual differentiation through disruption of AhR and AR signaling pathways

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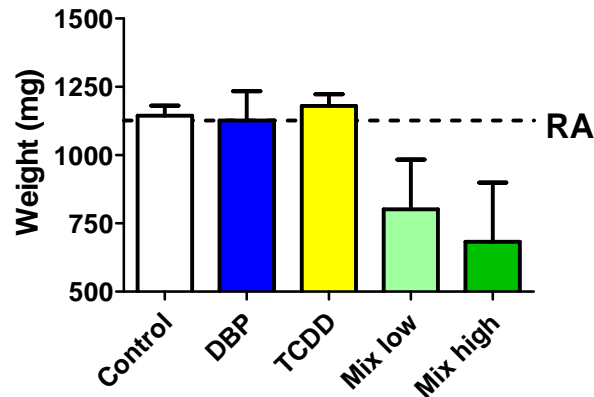
# DBP+TCDD malformations and organ weight reductions that exceeded Response Addition predictions

Bois et al. 2010

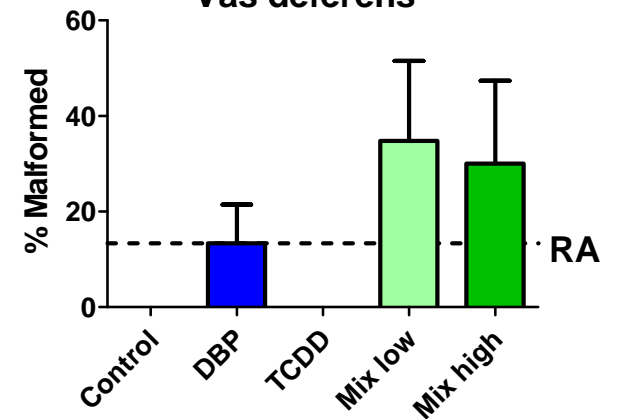
Epididymal and Testicular Malformations



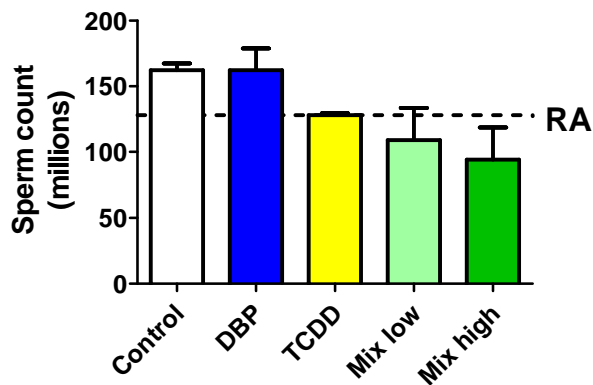
Epididymal Weight



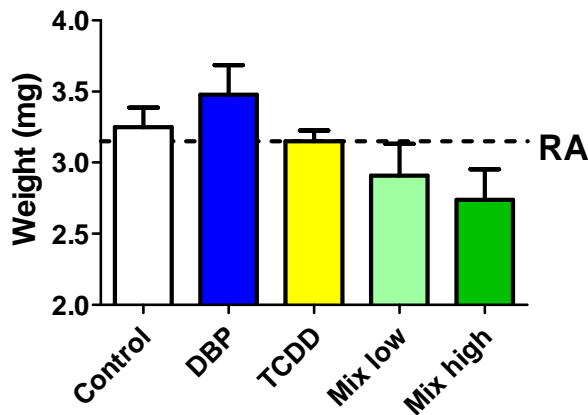
Agnesis of the Vas deferens



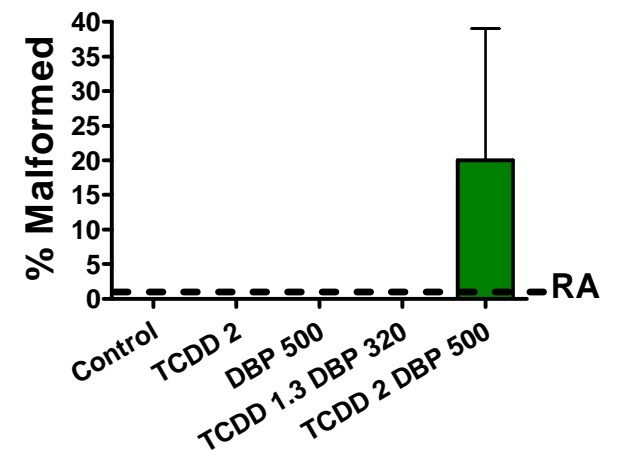
Epididymal Sperm Numbers



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 disrupted common reproductive tissues**
- **Differentiation of androgen-dependent tissues depends upon critical interactions of dynamic interconnected 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.**

# The "Team"

Dr. L. Earl Gray Jr.	USEPA
Dr. Vickie Wilson.	USEPA
Dr. Phillip Hartig.	USEPA
Johnathan Furr.	USEPA
Dr. Andrew Hotchkiss	USEPA
Christy Lambright	USEPA
Mary Cardon	USEPA

Dr. Cynthia Rider	Duke University
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Dr. Jerry LeBlanc	North Carolina State University
-------------------	---------------------------------

Dr Paul Foster	NIEHS
Dr. Chad Blystone	NIEHS
Dr. Kembra Howdeshell	NIEHS

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