NTP
National Toxicology Program

Disruption of Male Reproductive Development by Phthalate Esters

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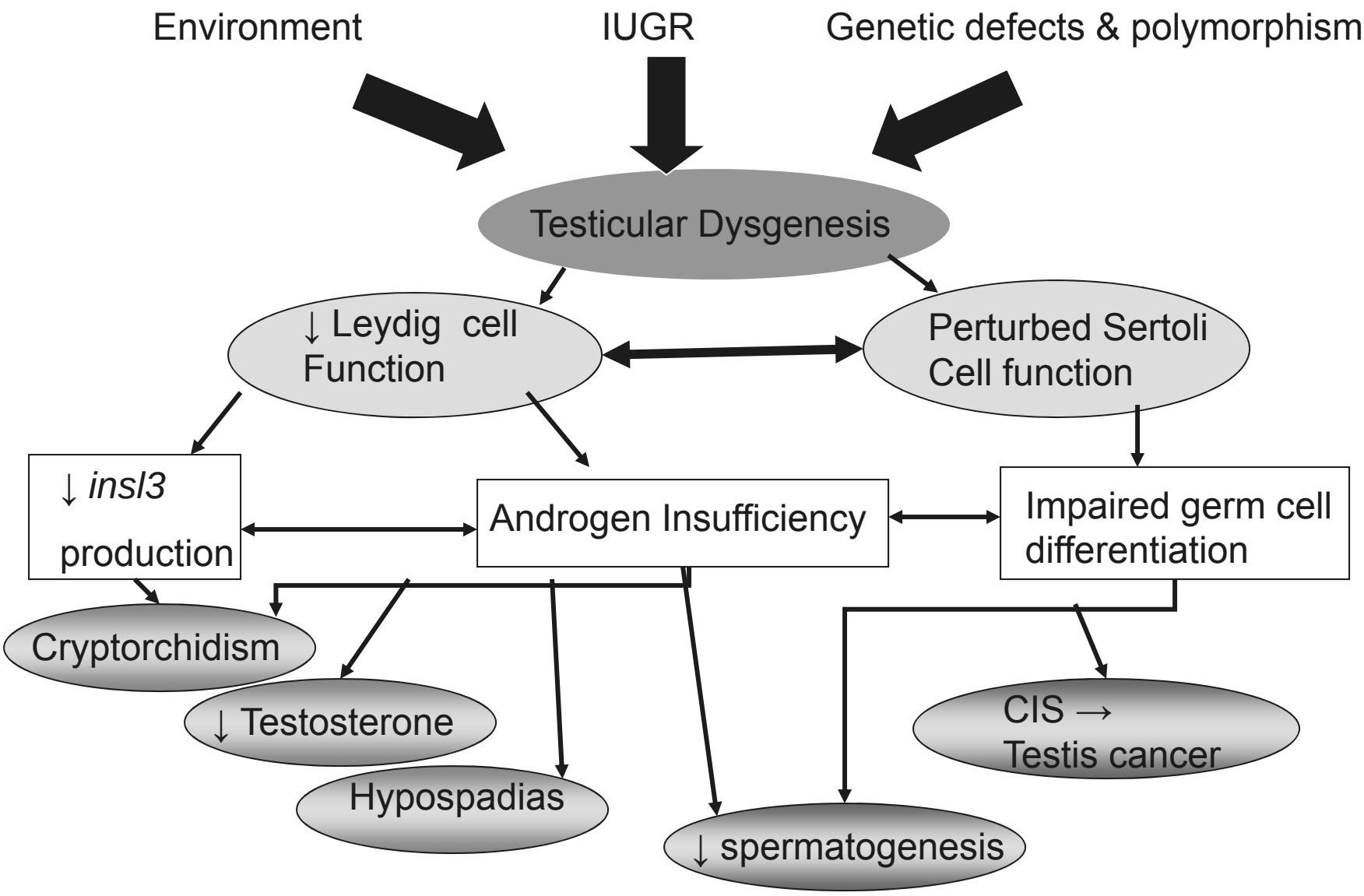
Presentation to CPSC CHAP July 2010

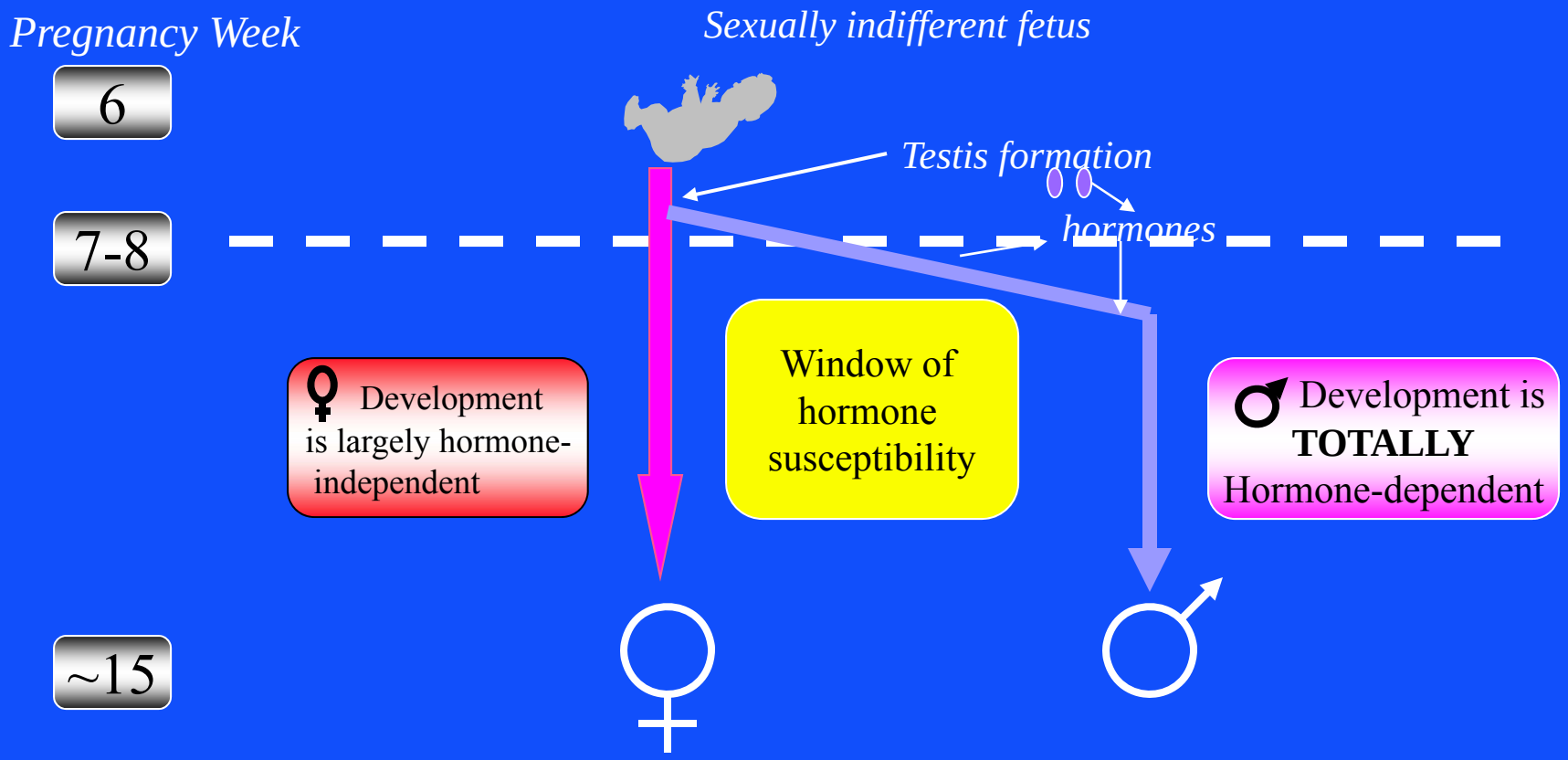
Overview

- Provide some background to disruption of male reproductive development in mammals.
- Brief background to the reproductive toxicity of *o*-phthalates.
- Describe the phenotypic pattern of lesions noted in male rat offspring following *in utero* exposure to di-*n*-butyl phthalate (DBP).
- Note the early changes in fetal testis testosterone production (and gene expression) as precursors to the adverse effects noted in the adult.
- Describe the critical windows for development of lesions in rats.

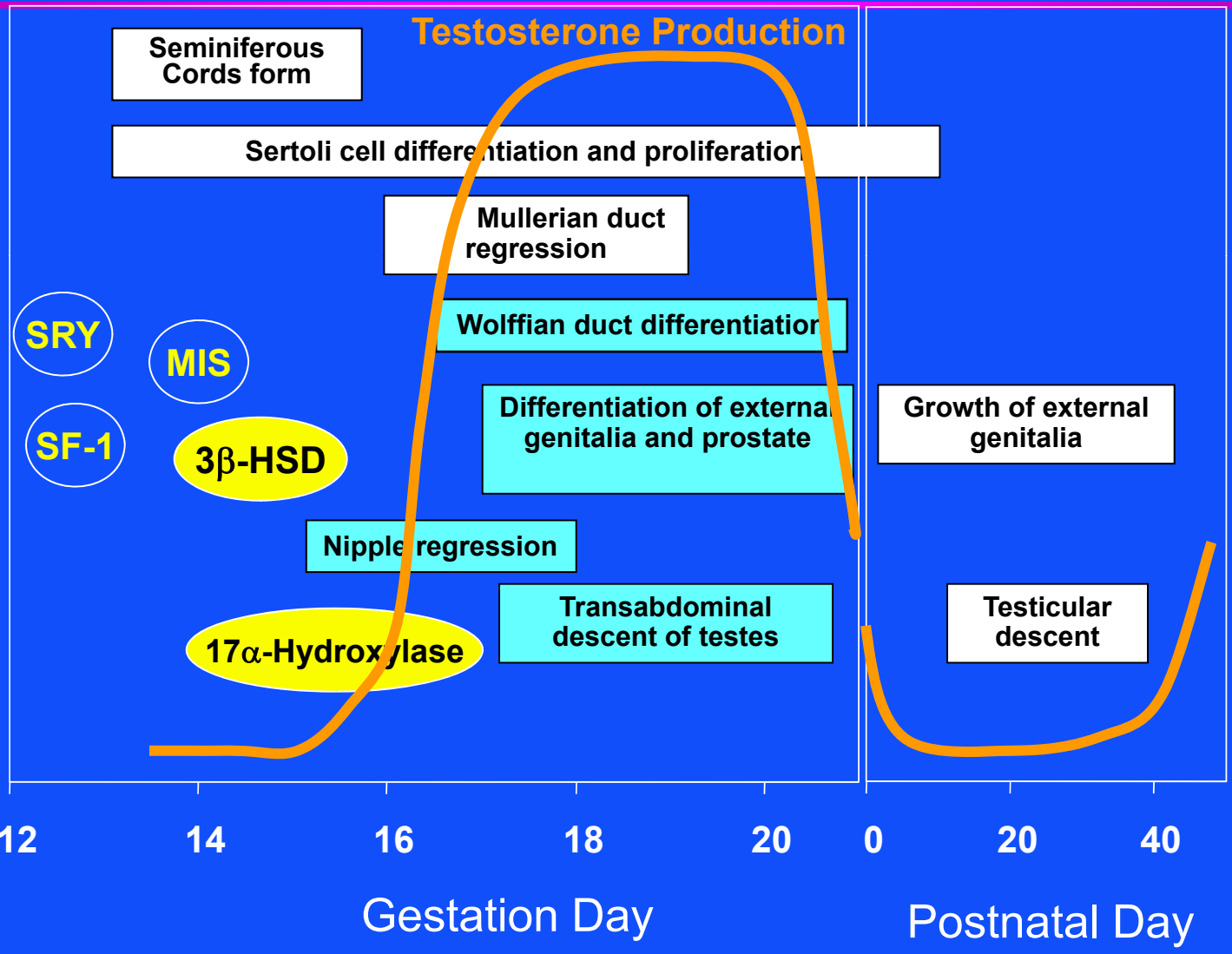
Human Testicular Dysgenesis Syndrome



Critical Window of Susceptibility: Male or Female?



Development of the Rat Male Reproductive Tract



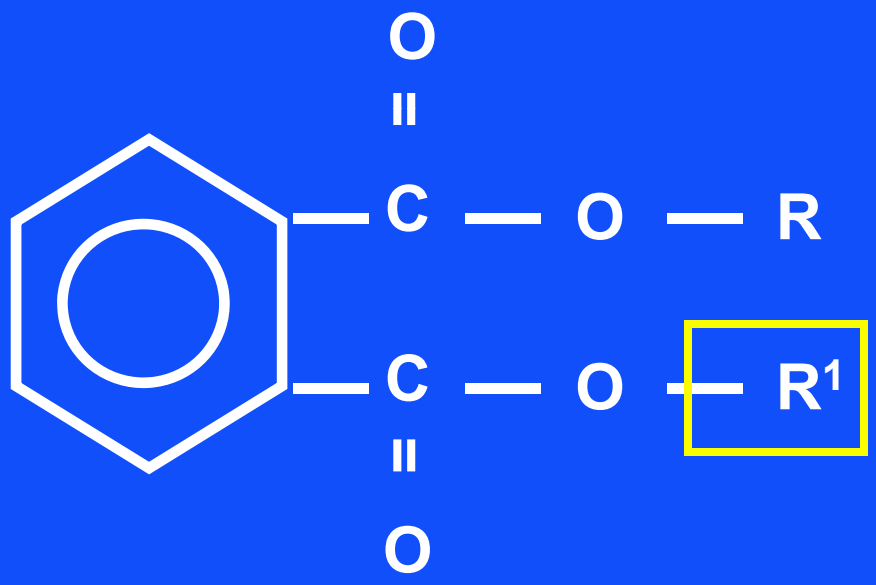
Phthalate Esters - Background

- Used extensively as a plasticizers and solvents.
- Some are weak ER agonists *in vitro* in some systems. No *in vivo* estrogenic activity.
- Generally not teratogenic in classical gd 6-15 studies in rats at levels <1g/kg/d. When developmental toxicity noted, always accompanied by maternal toxicity.
- Extensive metabolism occurs in the gut of rodents to form monoesters which are absorbed and believed responsible for reproductive and developmental toxicity.

Phthalate Esters - Background - 2

- Target Sertoli cells in the testes of pubertal and adult rats (FSH mechanism?)
- Age sensitivity in response (and likely different mechanisms):
  - Fetal>neonate>pubertal>adult
- Even though testicular toxicity has been known for > 25 years, only a limited number of multigeneration reproduction studies conducted.
- Effects are noted in females but tend to occur at higher dose levels than males.

Phthalate Esters known to induce effects on male reproductive development in rodents



- R=R^1= *n*-Butyl
  DBP
- R=R^1= *iso*-Butyl
  DiBP
- R=R^1= *n*-Pentyl
  DPP
- R=R^1= 2-ethylhexyl
  DEHP
- R=R^1= isononyl (branched)
  DINP
- R=R^1= cyclohexyl
  DCHP
- R= n-Butyl R^1= benzyl
  BBP

- Likely follows SAR for pubertal testis effects - Foster *et al* (1980) TAP 54:392-8.

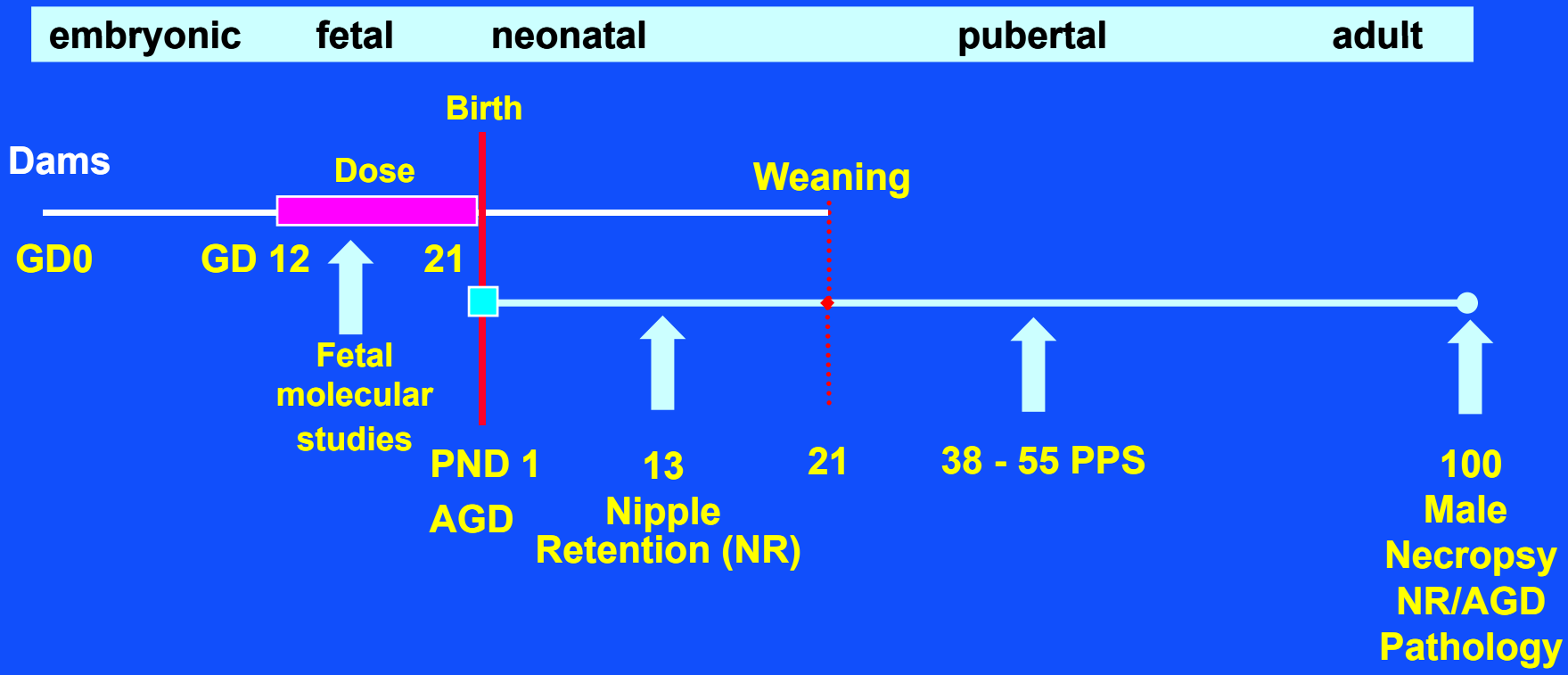
Effect of some phthalate esters on germ cell detachment in rat testicular cell cultures

Phthalate Monoester	Testicular Toxicity	No. of germ cells detached (%of Control) at (μM)					
		1	10	100	1000	2000	10000
2-Ethylhexyl	YES	208 *	213 *	384 *			
n-Heptyl	YES	130 #	161 #	500 +			
n-hexyl	YES	122 +	199 *	231			
n-pentyl	YES	111	147	206	276 *		
n-butyl	YES		113	143	165 +	228 *	
Tert-butyl	NO				115	110	307 *
n-propyl	NO				100	99	259 *
Ethyl	NO				86	81	210 *
Methyl	NO				103	86	48 *

After Foster et al (1980) and Gray& Beamand (1984)

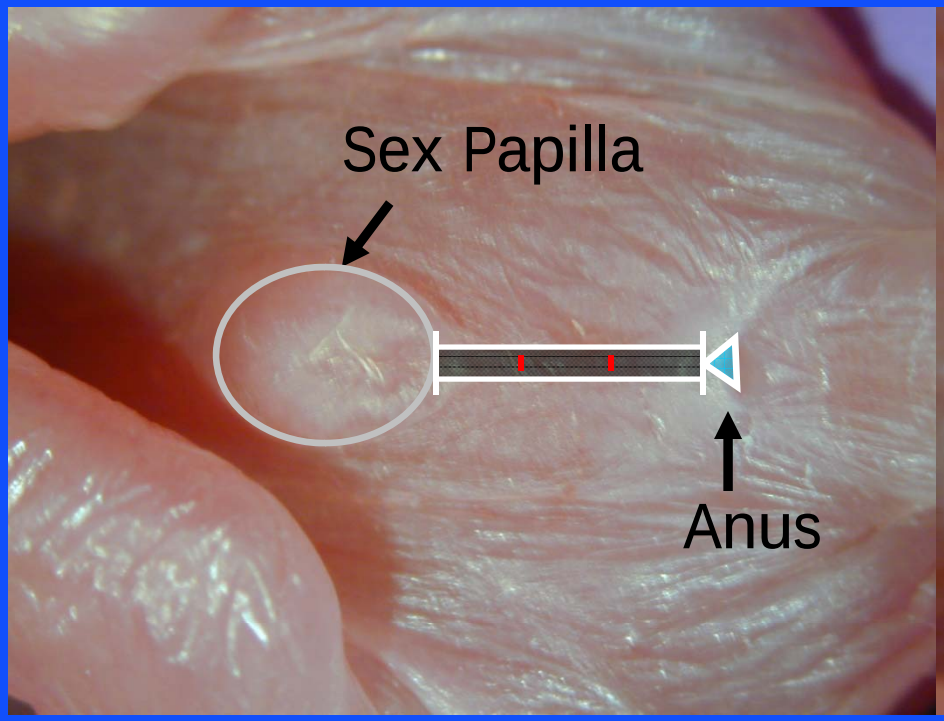
* $p<0.001$; # $p<0.01$; + $p<0.05$

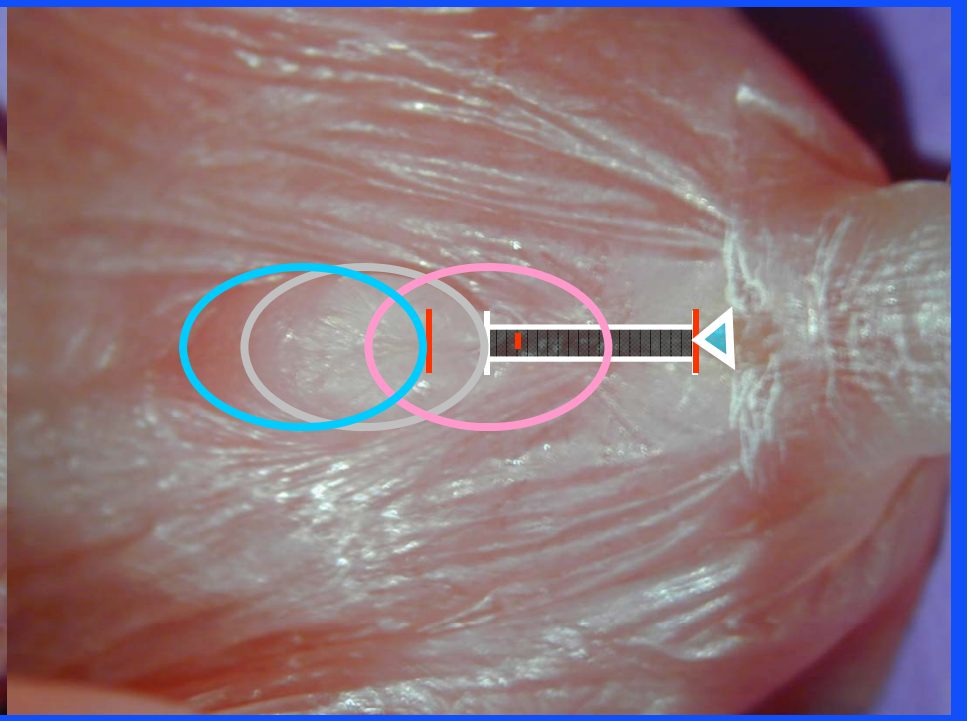
General Study Design



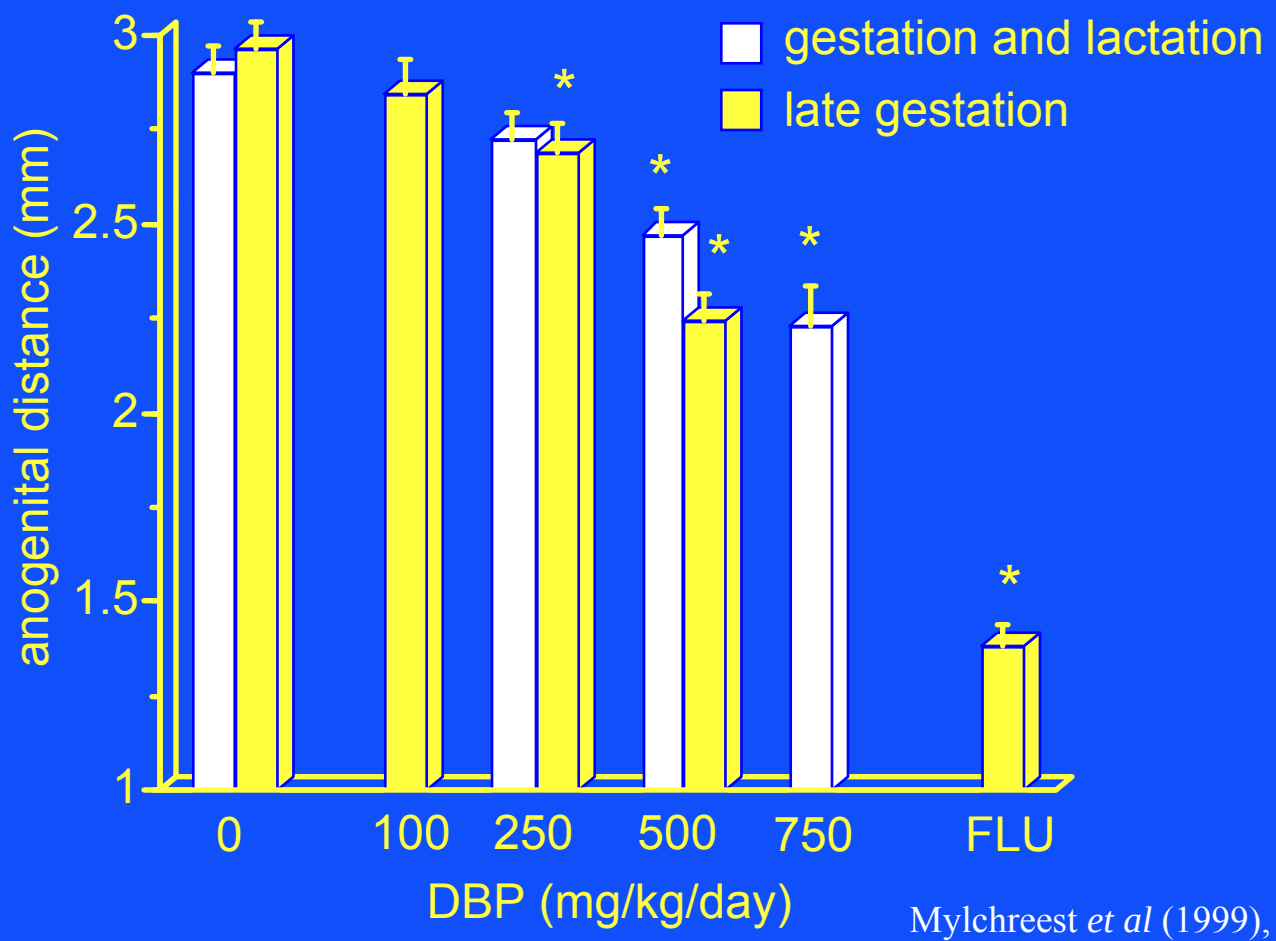
***All male pups retained to adulthood;**
Dose levels to produce minimal or no dam toxicity
DBP up to 500mg/kg/d

Anogenital Distance



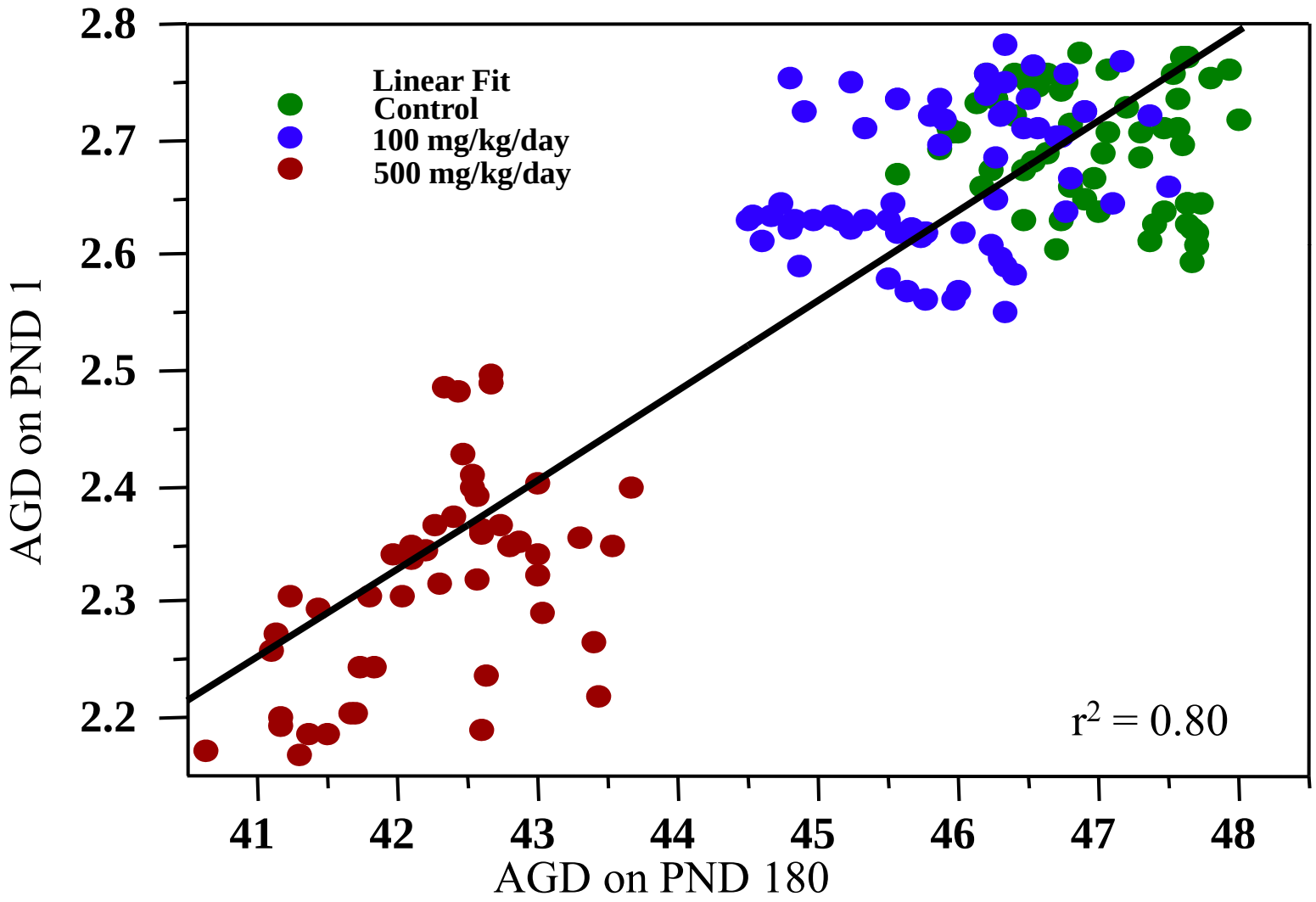


DBP "Feminizes" Anogenital Distance

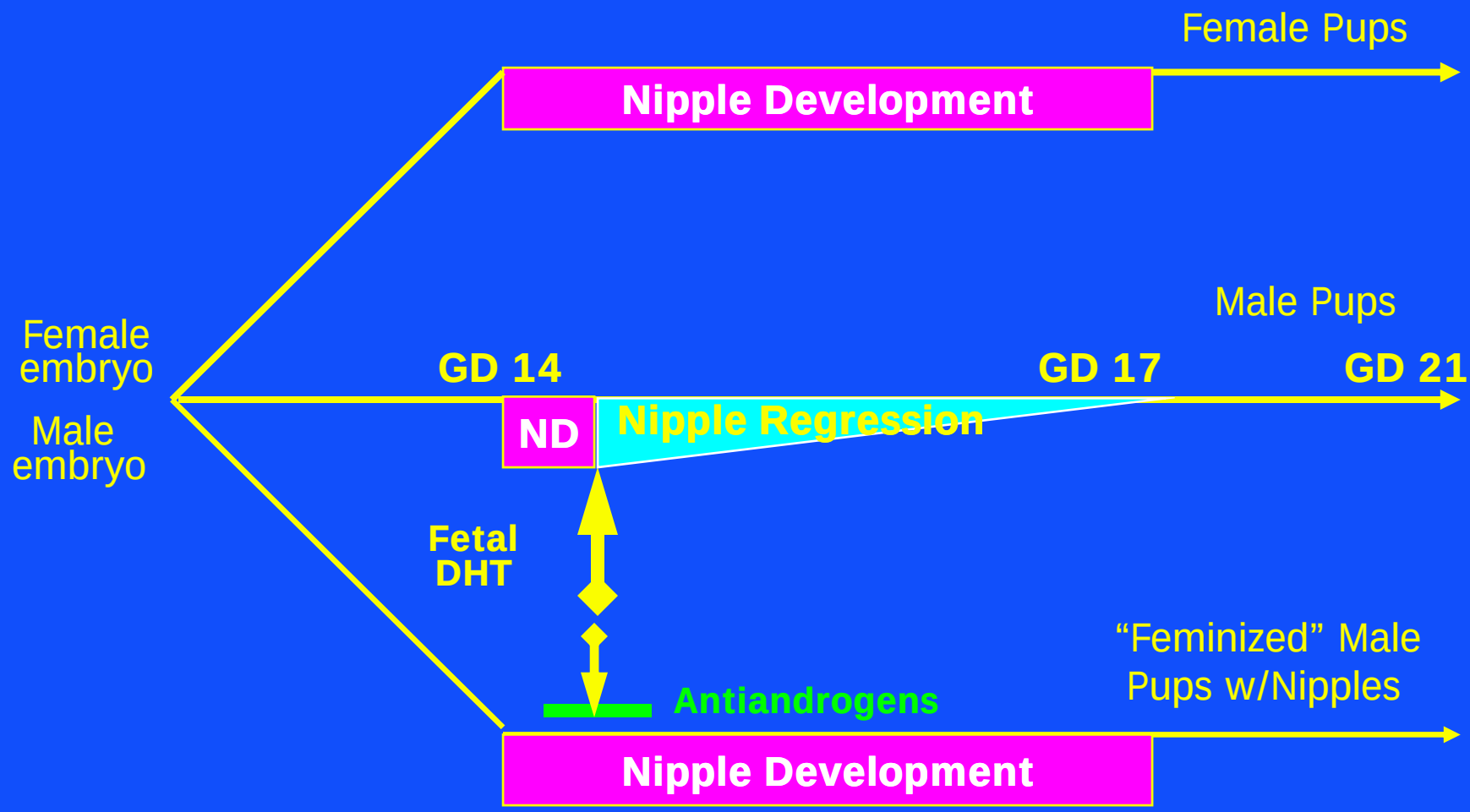


Mylchreest *et al* (1999), *TAP* **156**:81-95

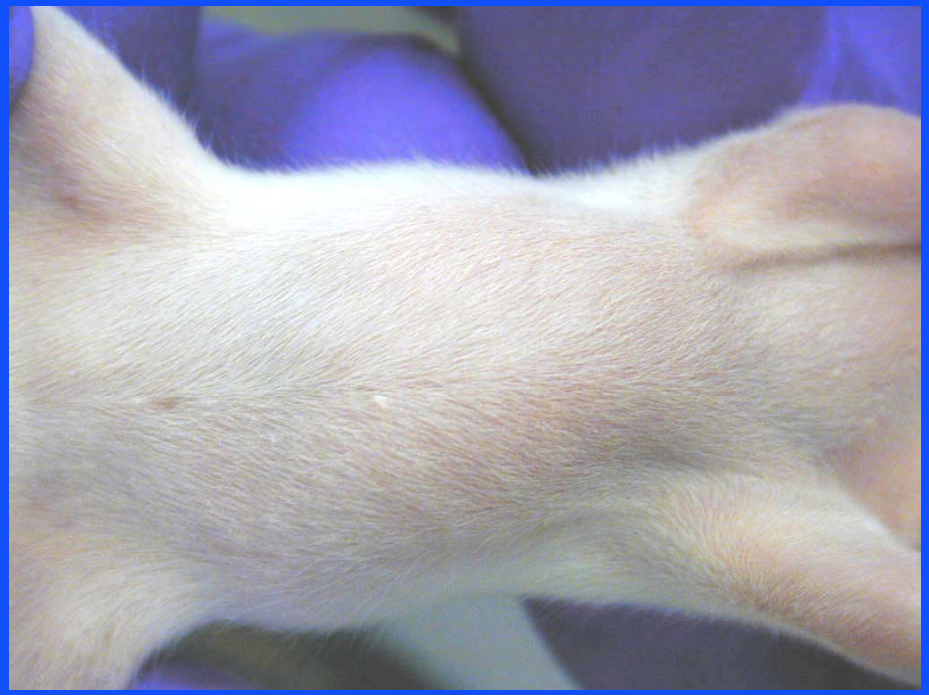
Permanent Effects on AGD (mm) after *in utero* exposure to DBP

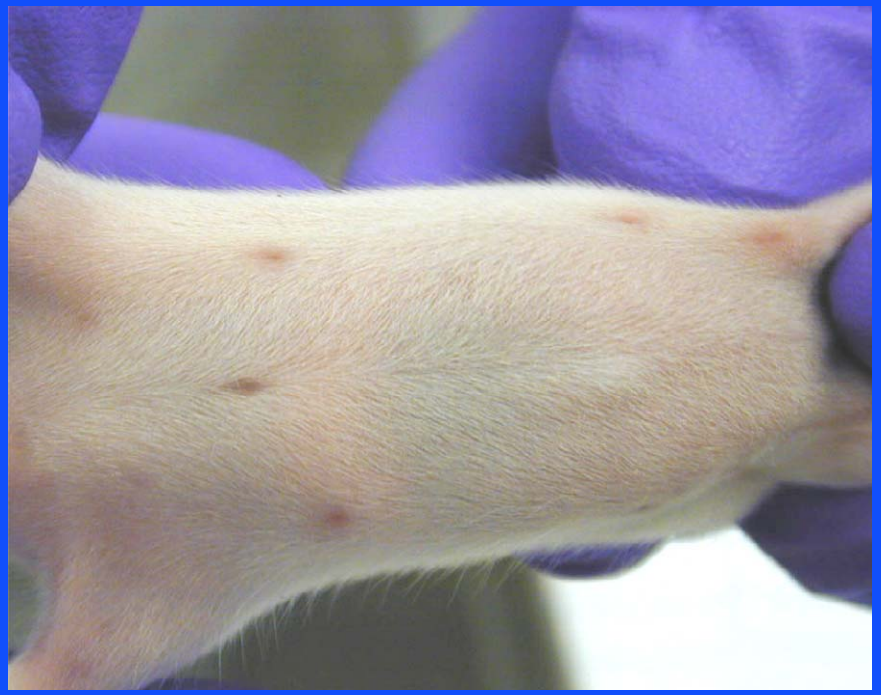


Nipple Development in the Rat

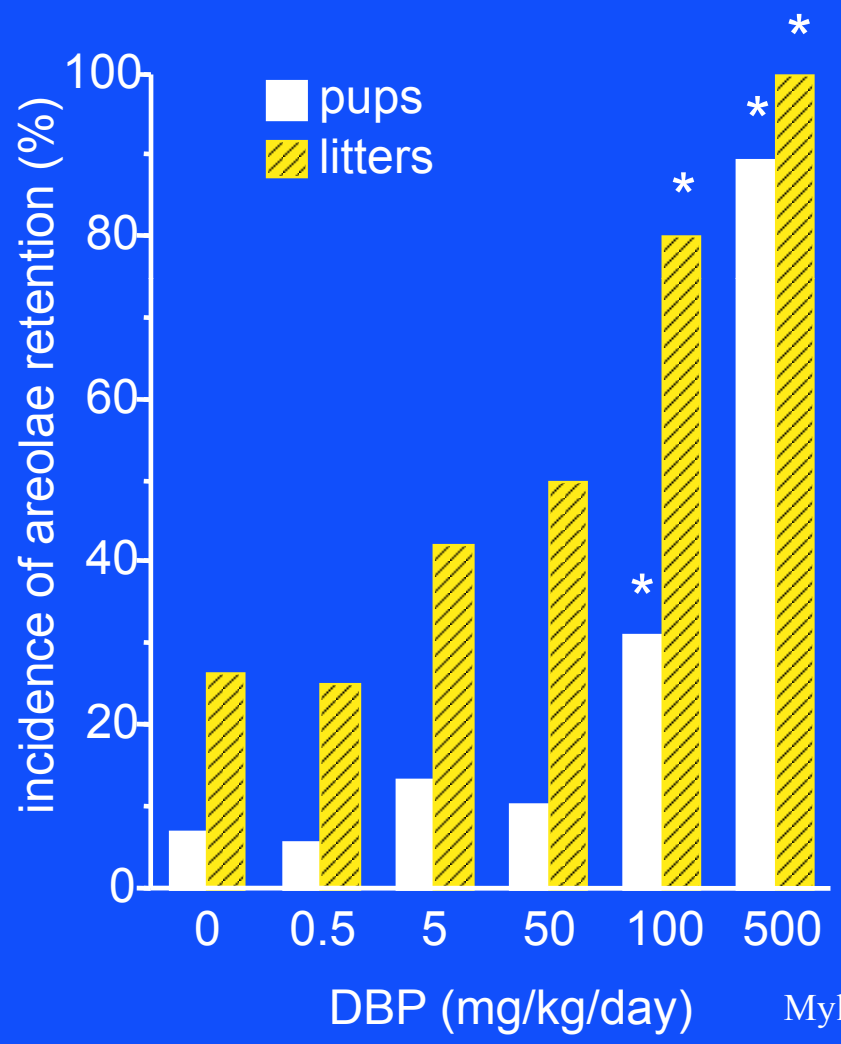


Areolae-Nipple retention on PND 13





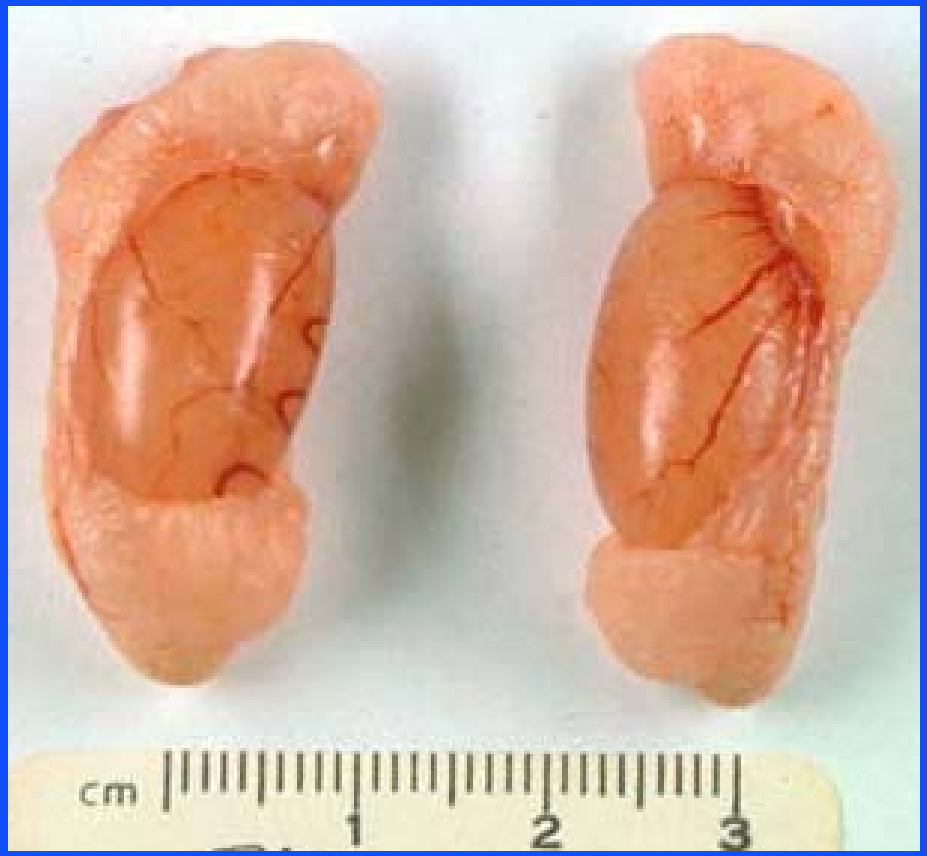
DBP Induces Areolae Retention in PND 13 rats



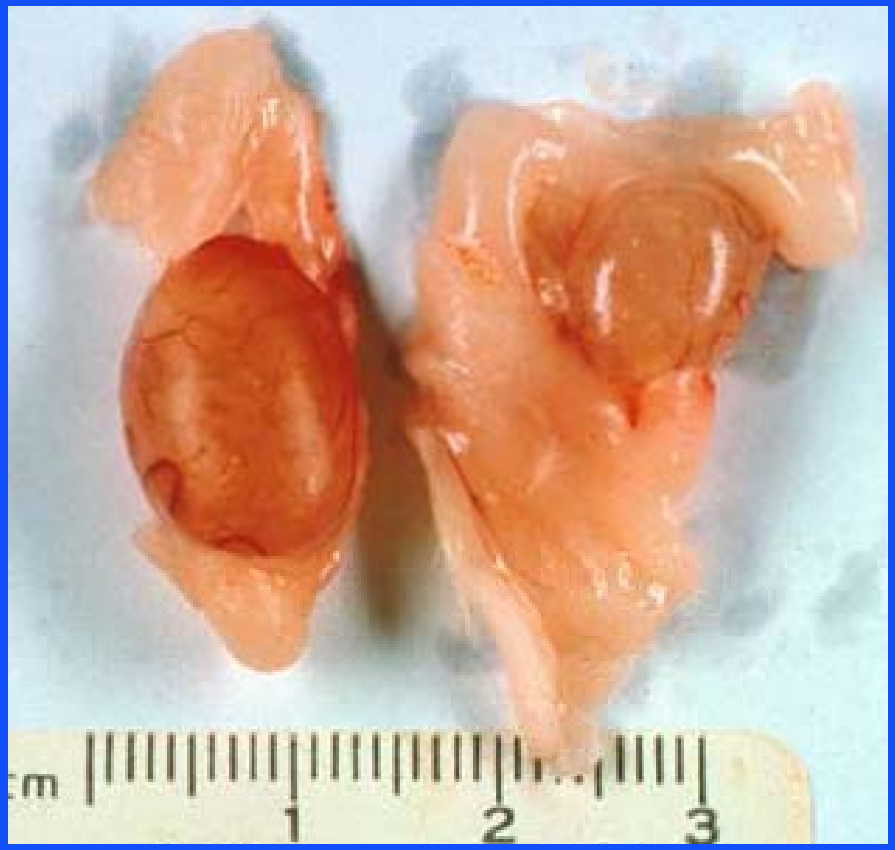
Mylchreest *et al* (2000), *Tox Sci* **55**:143-151

Testis & Epididymis following *in utero* DBP exposure

Control

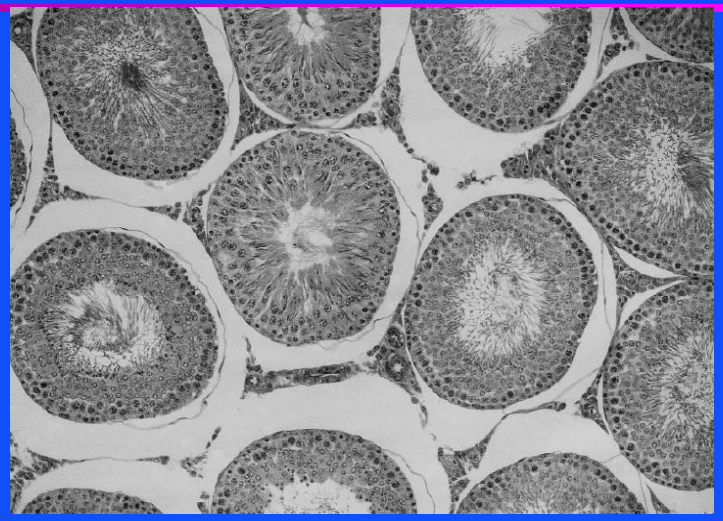


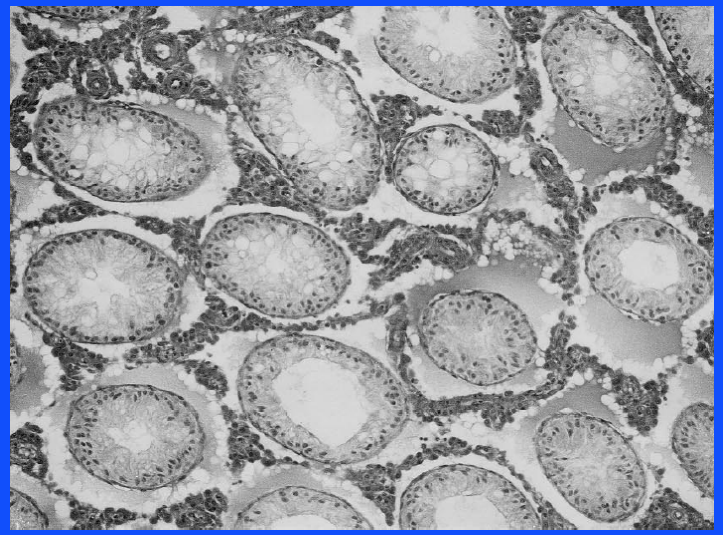
DBP



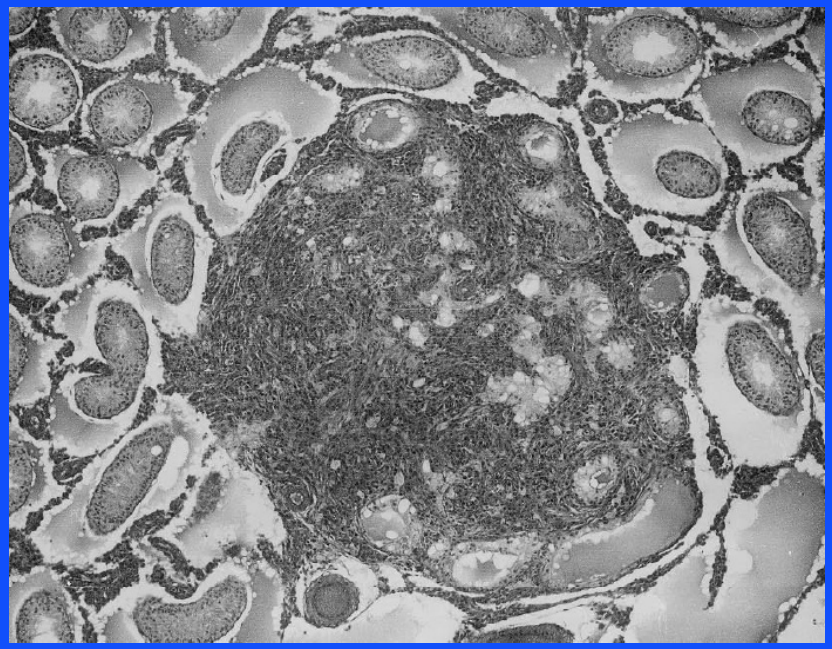
Mylchreest *et al* (1998), *Tox Sci* **43**:47-60

Adult testicular lesions following *in utero* DBP exposure





Control



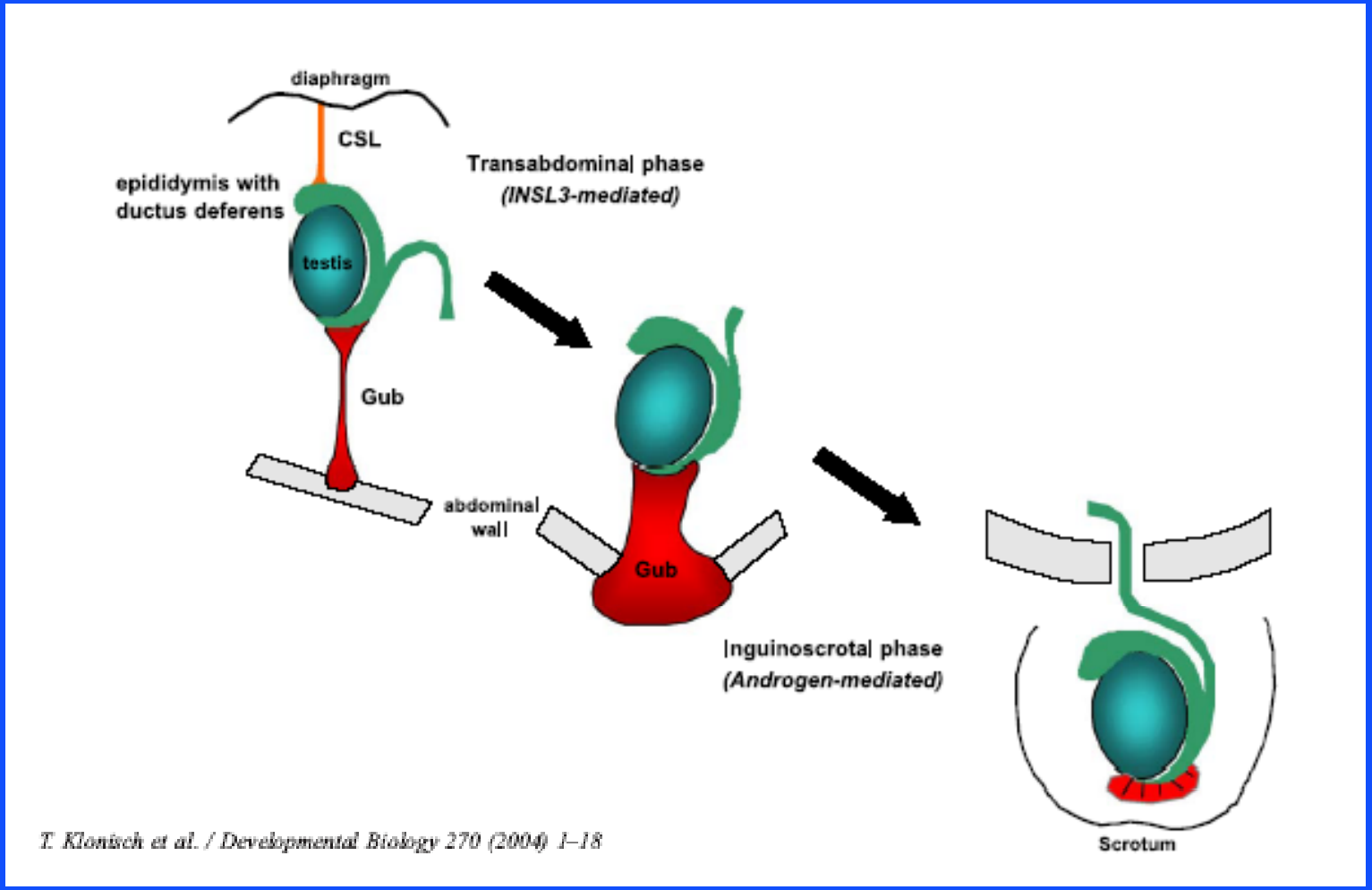
Leydig cell adenoma

DBP

Mylchreest *et al* (1999), *TAP* **156**:81-95

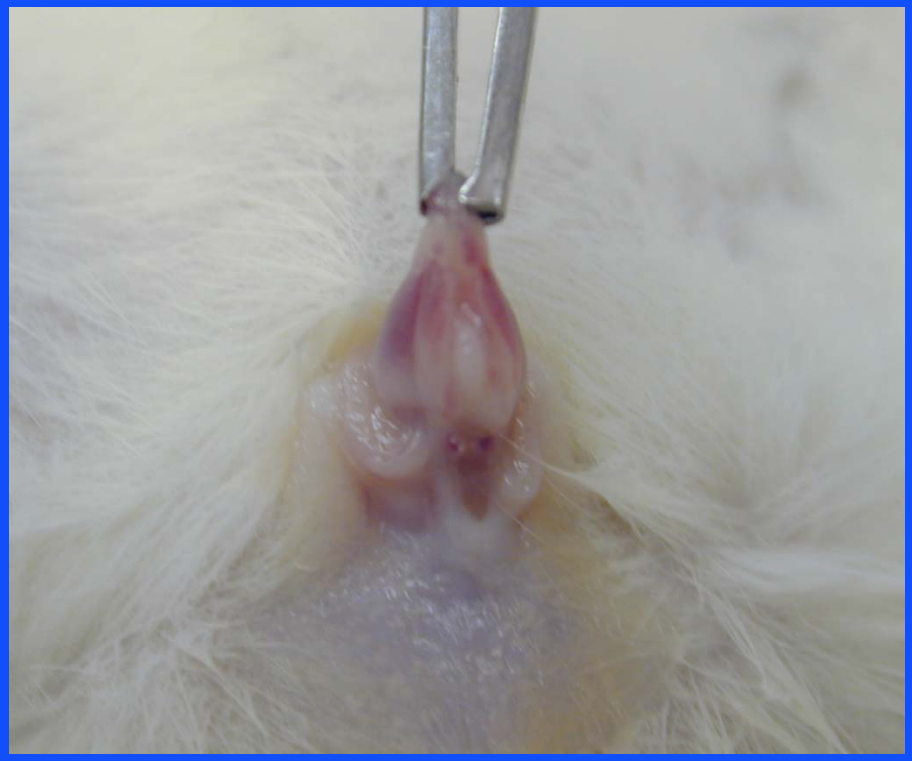
DBP – induced Cryptorchidism

Testicular Descent



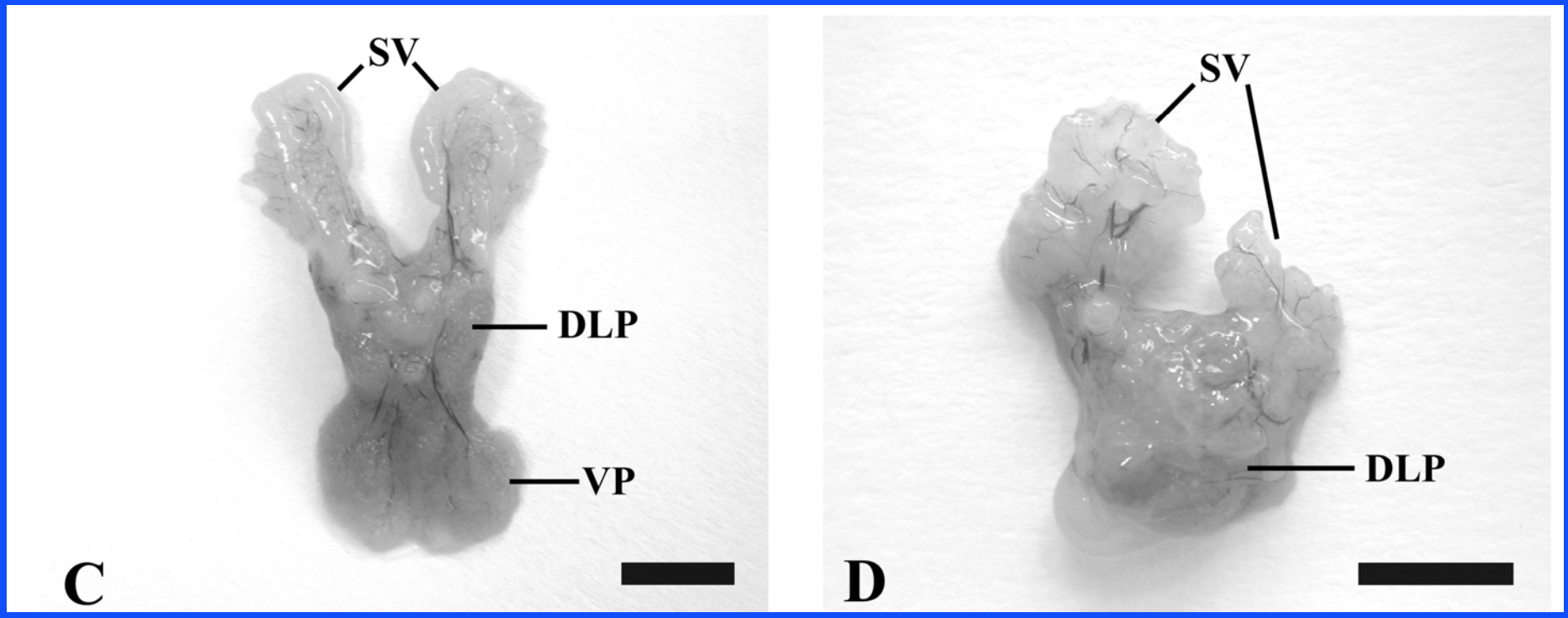
DBP-Induced Hypospadias

Control



DBP

Prostate and Seminal Vesicles after *in utero* DBP exposure

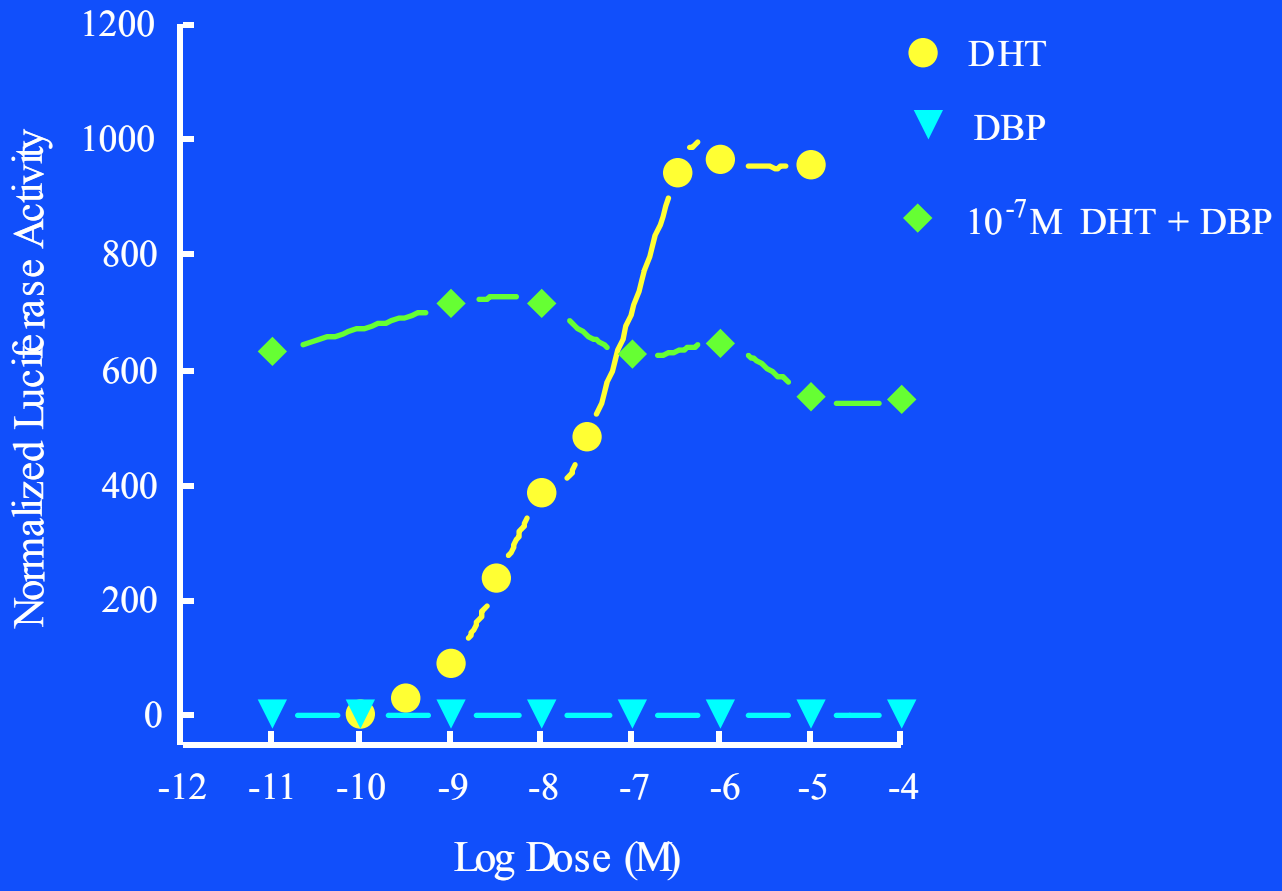


Control

DBP

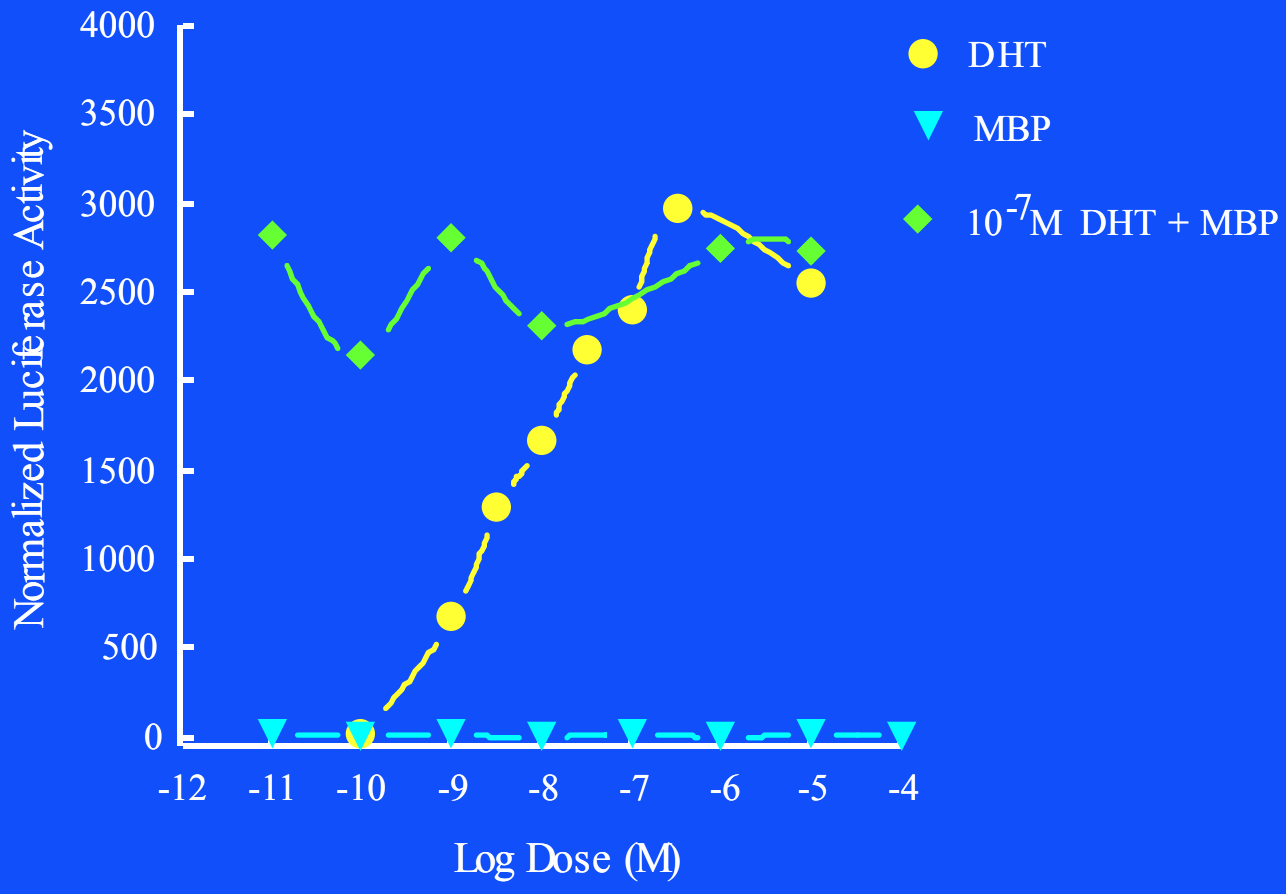
Bar = 1.0 cm

DBP Does Not Interact with the Androgen Receptor



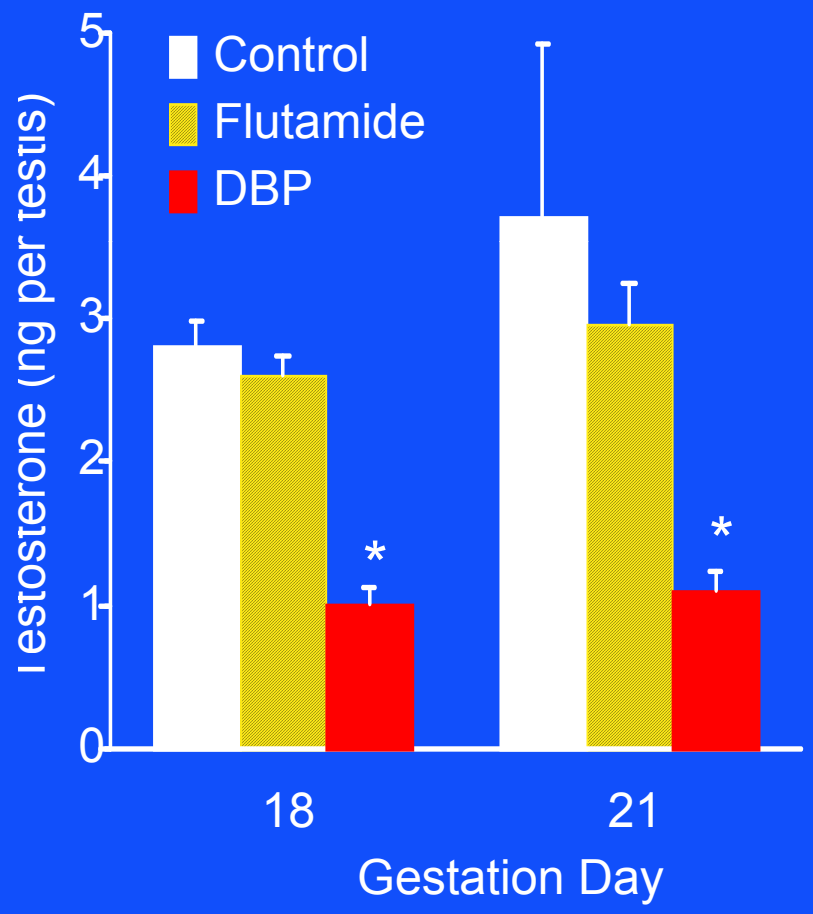
Foster et al (2001) *Human Reprod Update* 7: 231-235

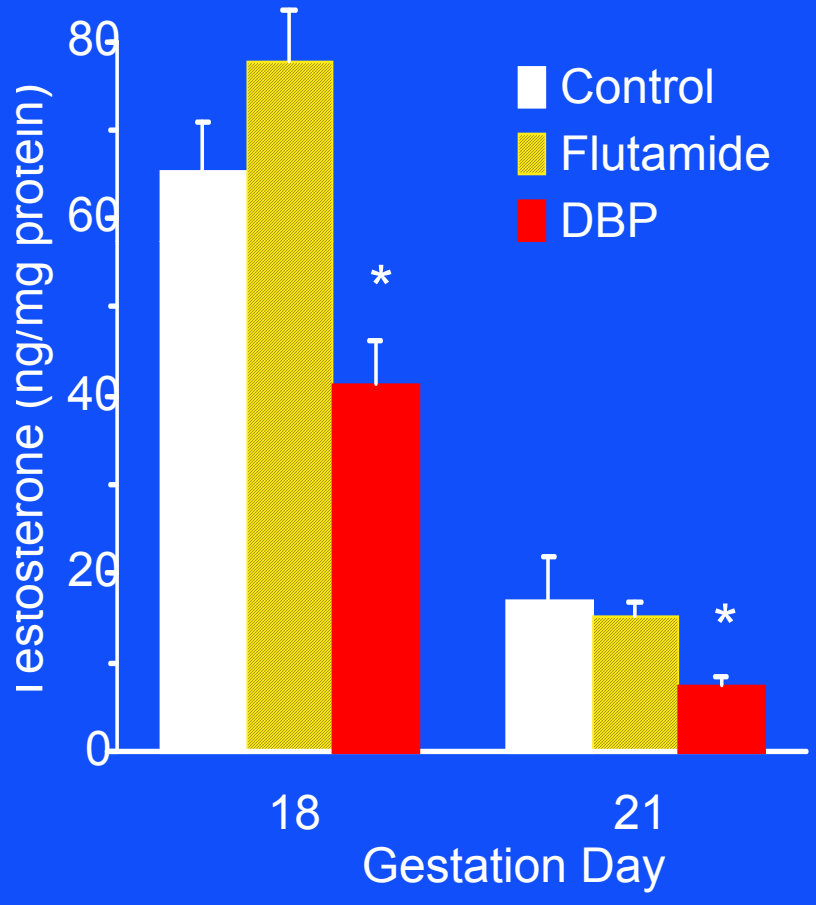
AR Assay with Mono-*n*-butyl Phthalate (MBP)



Foster et al (2001) *Human Reprod Update* 7: 231-235

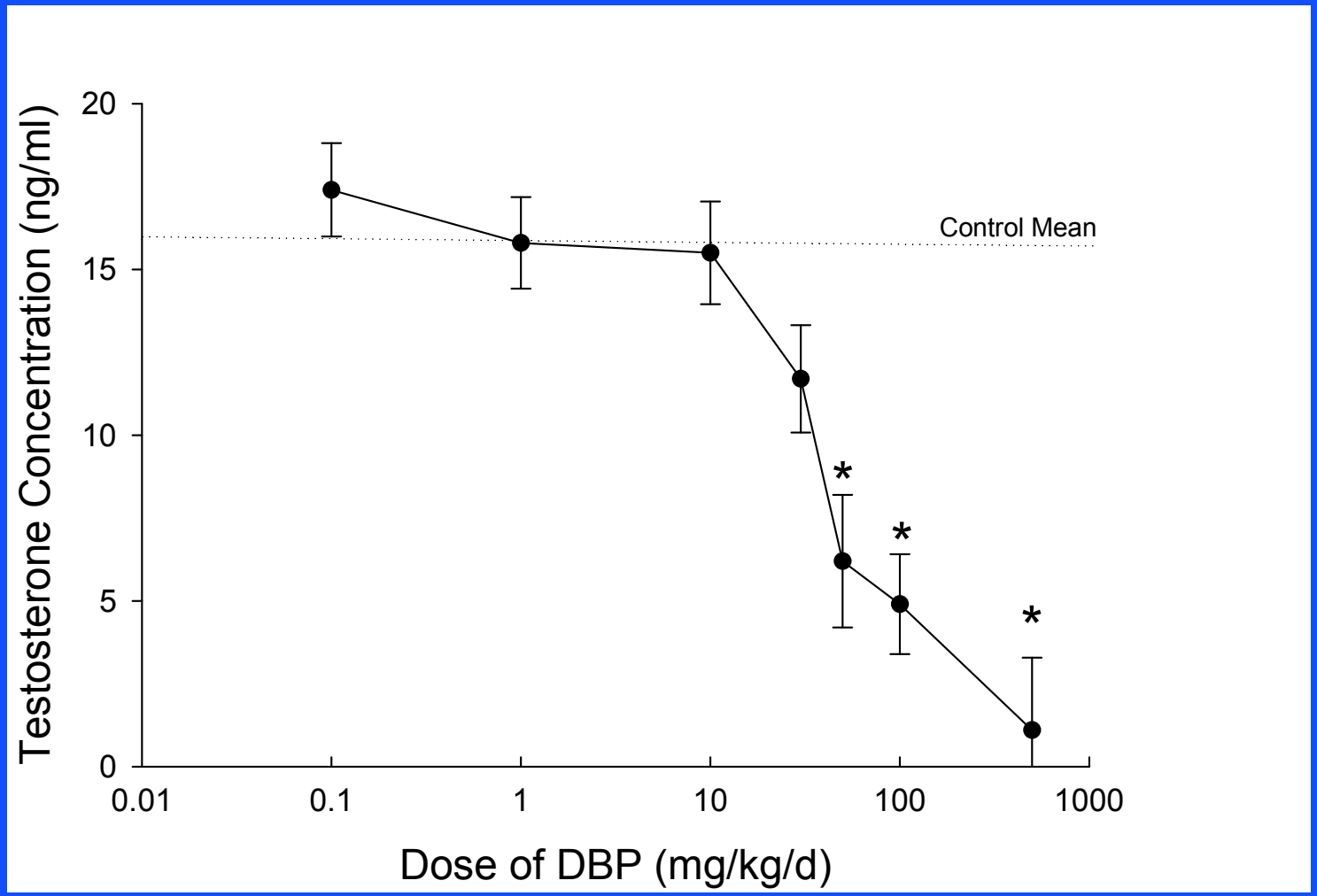
Fetal Testicular Testosterone





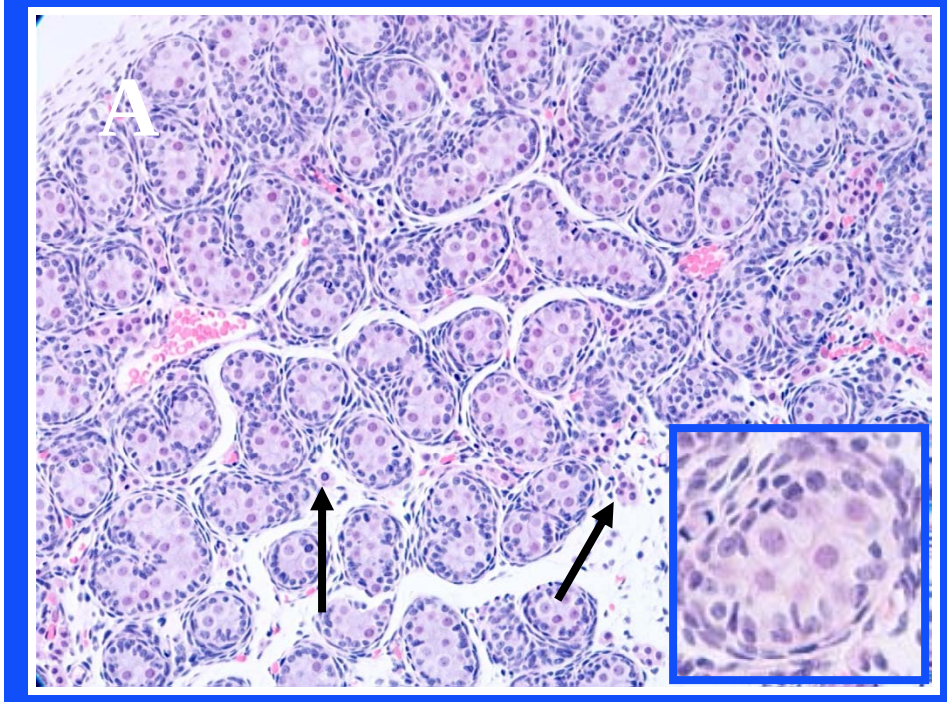
Mylchreest et al (2002) *Reprod Tox* 16:19-28

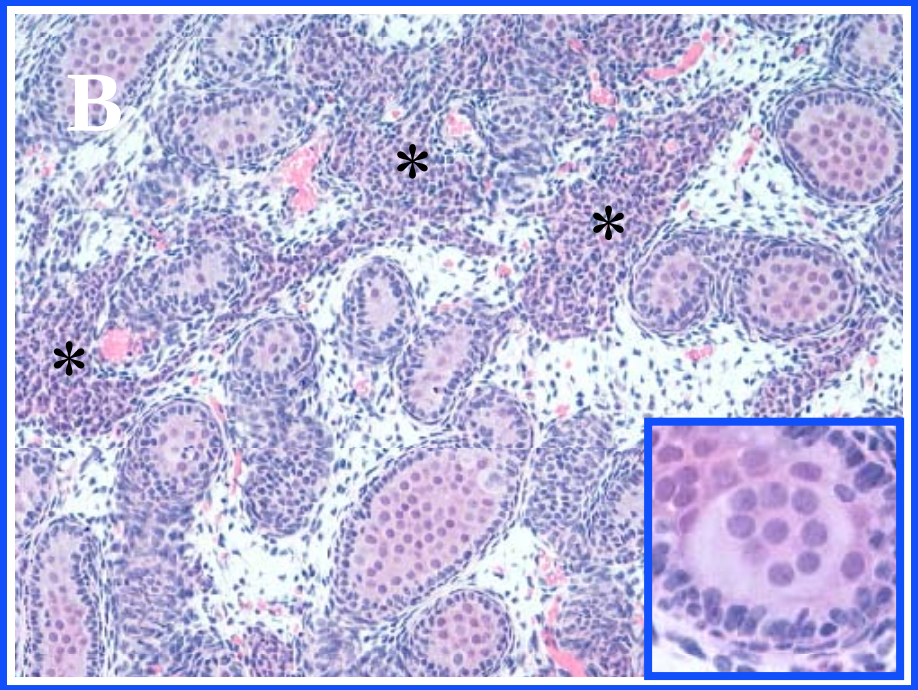
Fetal testicular testosterone (GD 19) after *in utero* DBP



Lehmann et al (2004) *Tox Sci* 81: 60-68

Interstitial Cell Aggregates and Multinucleate Gonocytes after DBP treatment - GD 21

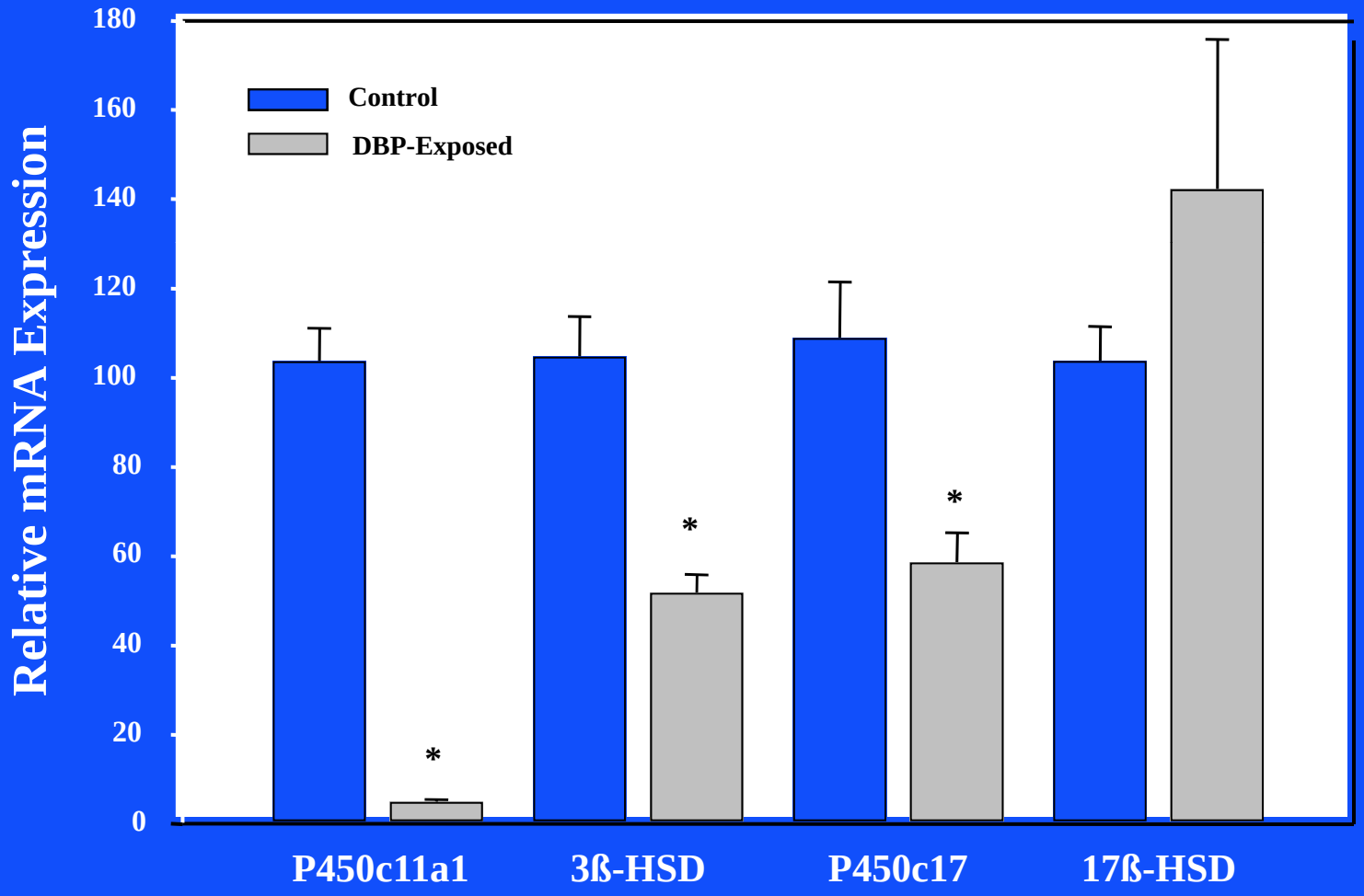




Control

DBP-treated

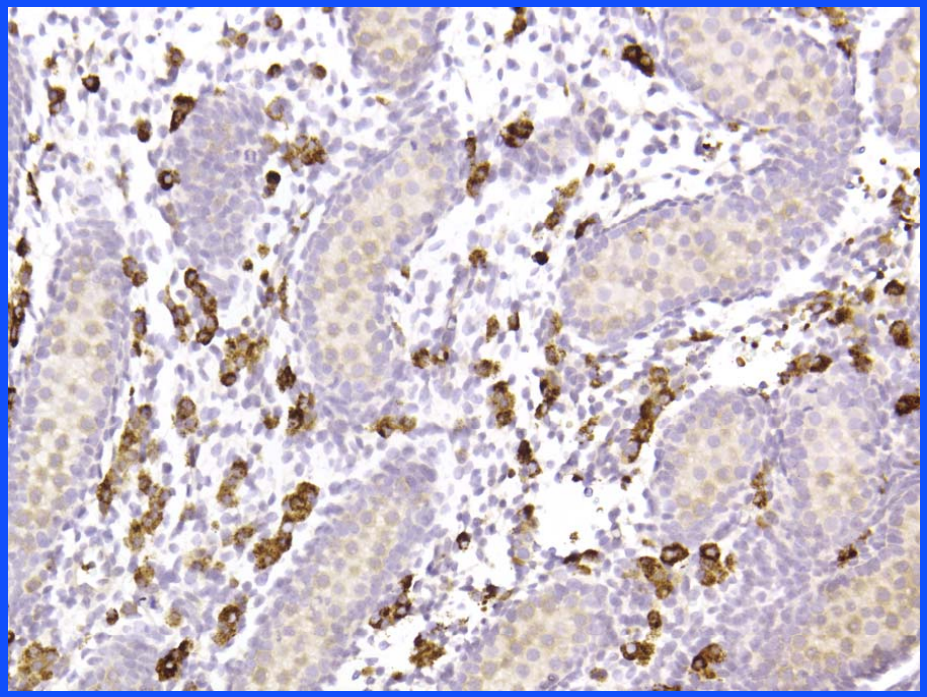
Gene Expression Changes in Steroidogenic enzymes in GD 19 Testes



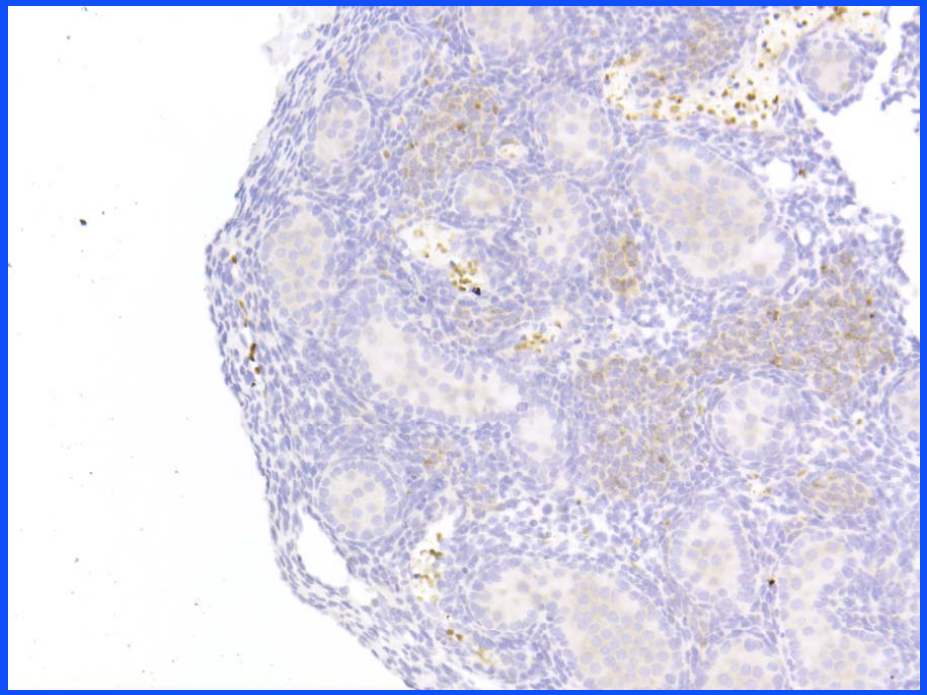
Litter means ± SE; 3♂ from at least 5 litters

Barlow *et al*. (2003) *Tox Sci* **73**, 431-441.

StAR Expression in GD 19 Fetal testes

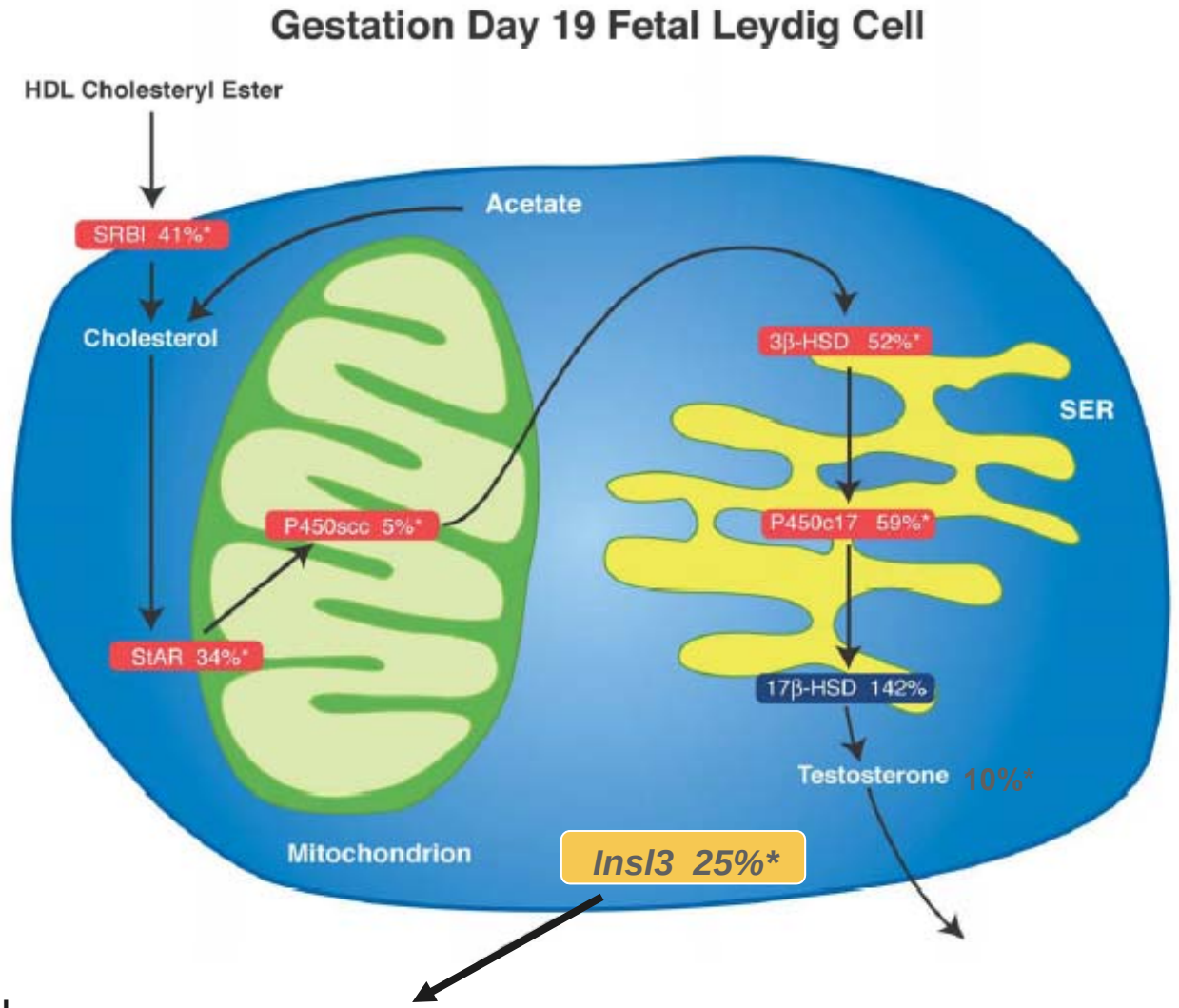


Control



DBP

Barlow *et al*. (2003) *Tox Sci* **73**, 431-441.

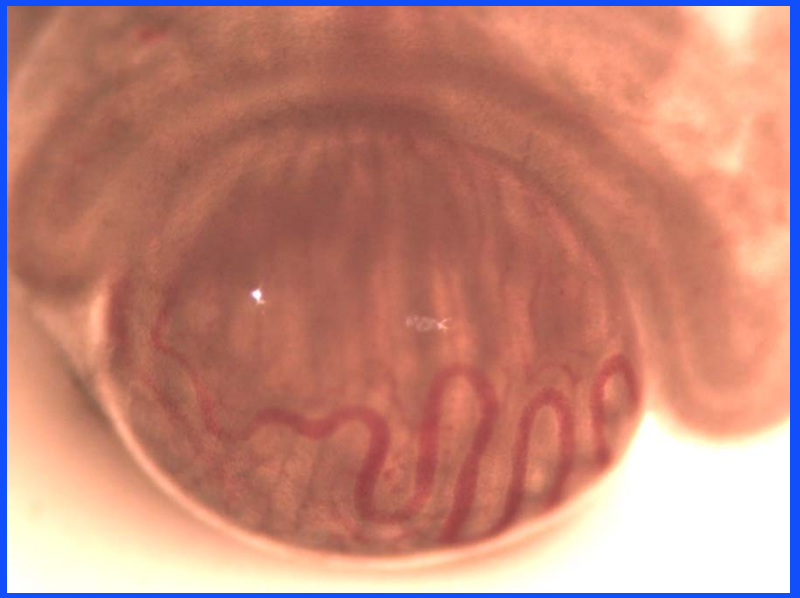


Values % control

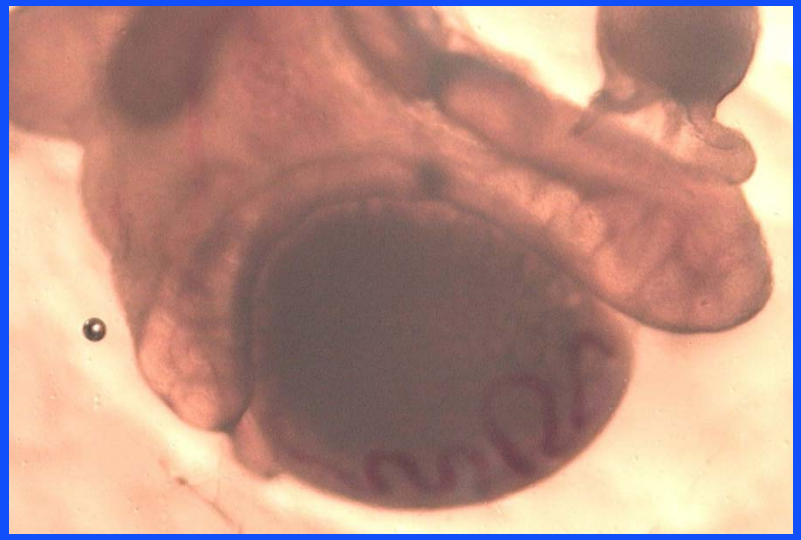
Foster (2006) *Int J. Androl* 29:140-147

GD 19 control

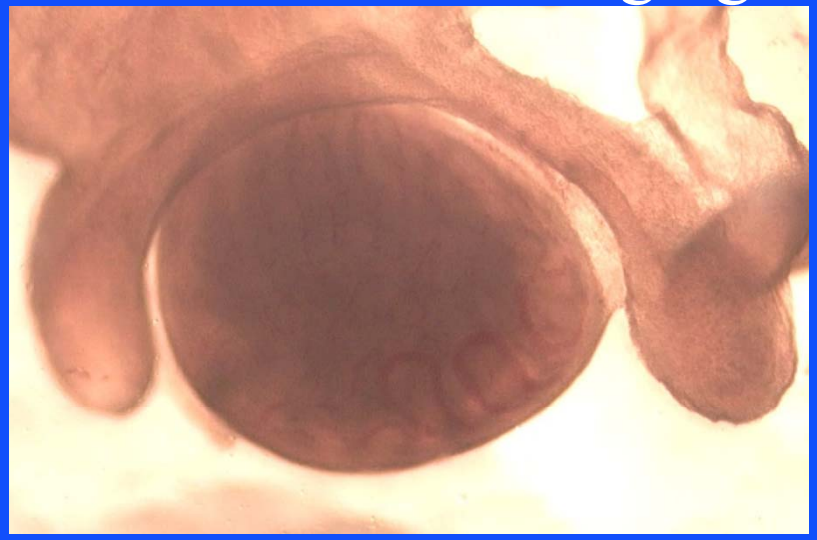
GD 19 DBP 500mg/kg



GD 21 control

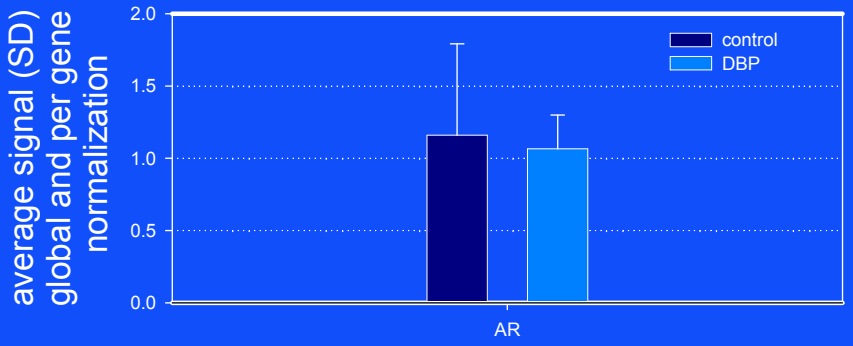


GD 21 DBP 500mg/kg

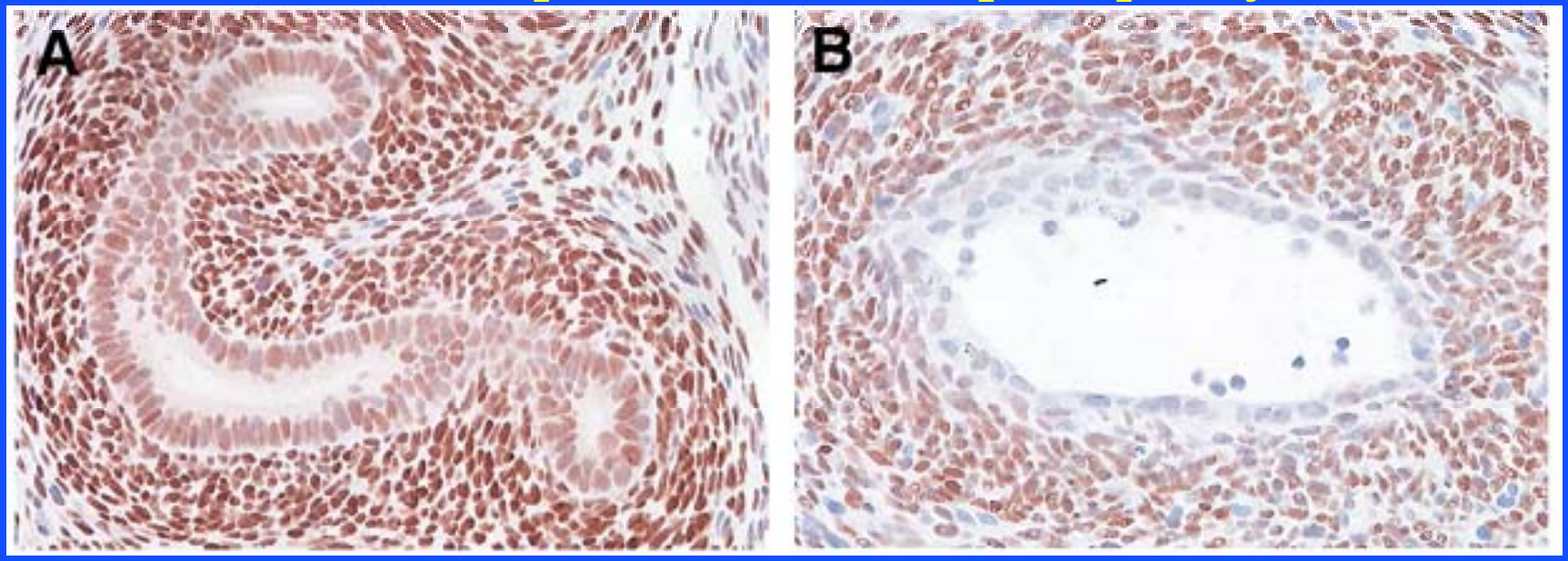


Androgen receptor

- Empirical determination
- Localization vs. tissue level



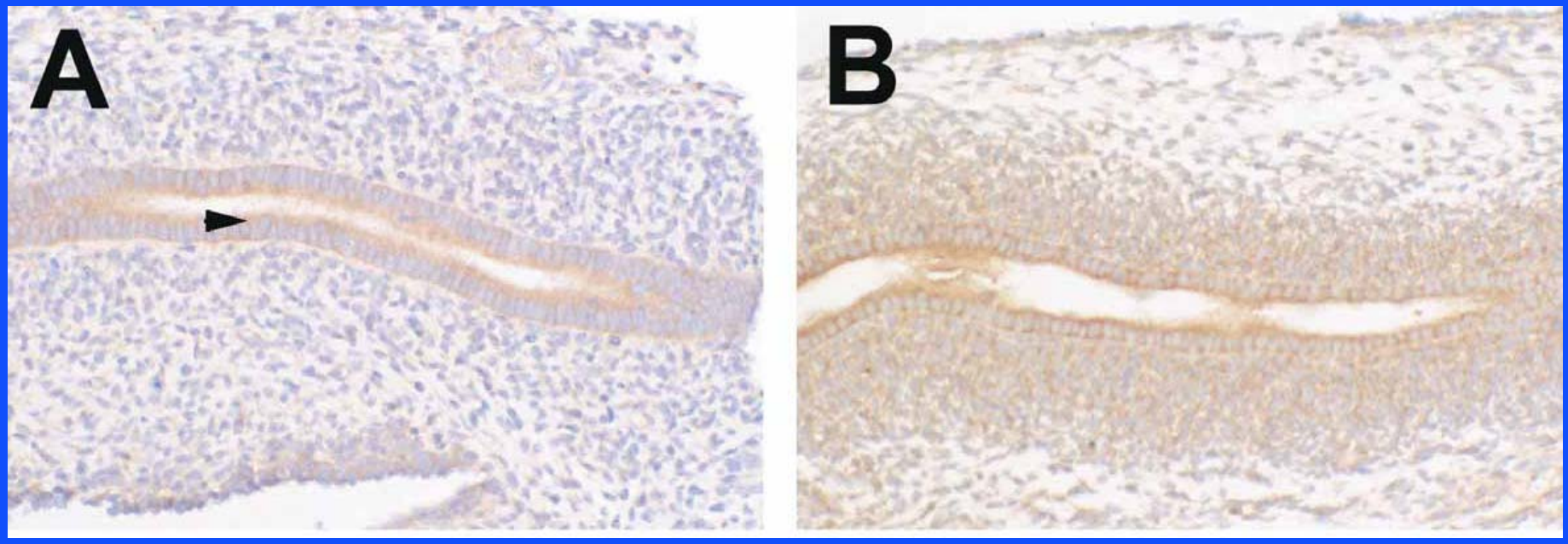
AR protein-GD 21 Caput Epididymis



Control DBP

Mylchreest et al (2002) *Reprod Tox* 16:19-28

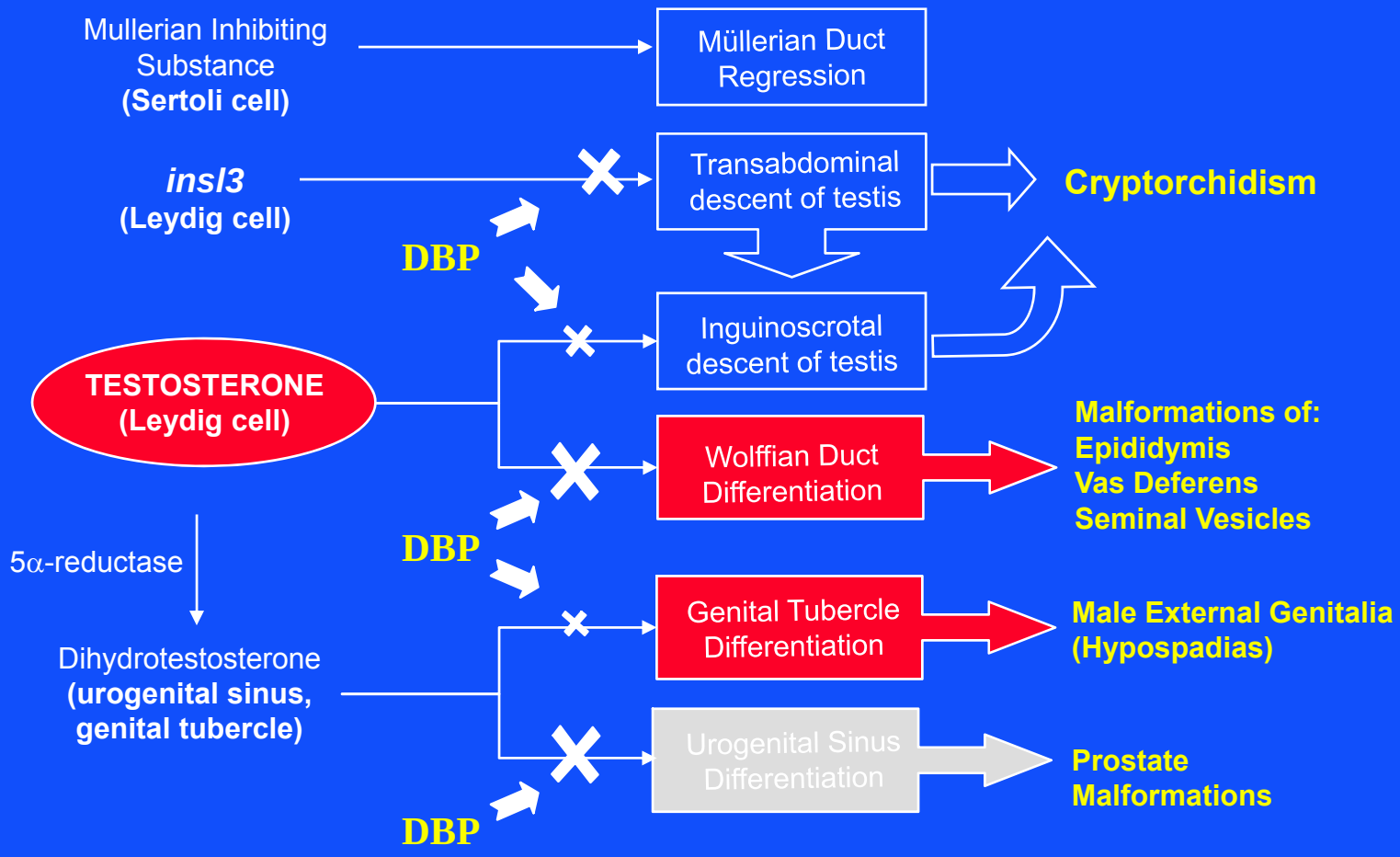
IGFR1 immunoexpression in Fetal Epididymis (GD 19)



Control

DBP

Schematic of the Effects of DBP on Male Reproductive Development – the "Phthalate Syndrome"



"Phthalate syndrome"

Biological Plausibility

Human Reproductive End Points of Concern with a Potential *In Utero* Origin.	**Effects Noted After *In Utero* Di(n-butyl)phthalate Exposure In Rats**
Infertility	✓
Decreased sperm count	✓
Cryptorchidism (Testicular maldescent)	✓
Reproductive tract malformations	✓
Hypospadias (penile malformation)	✓
Testicular tumors (germ cell)	✓ (Leydig cell adenoma; multinucleate gonocytes)

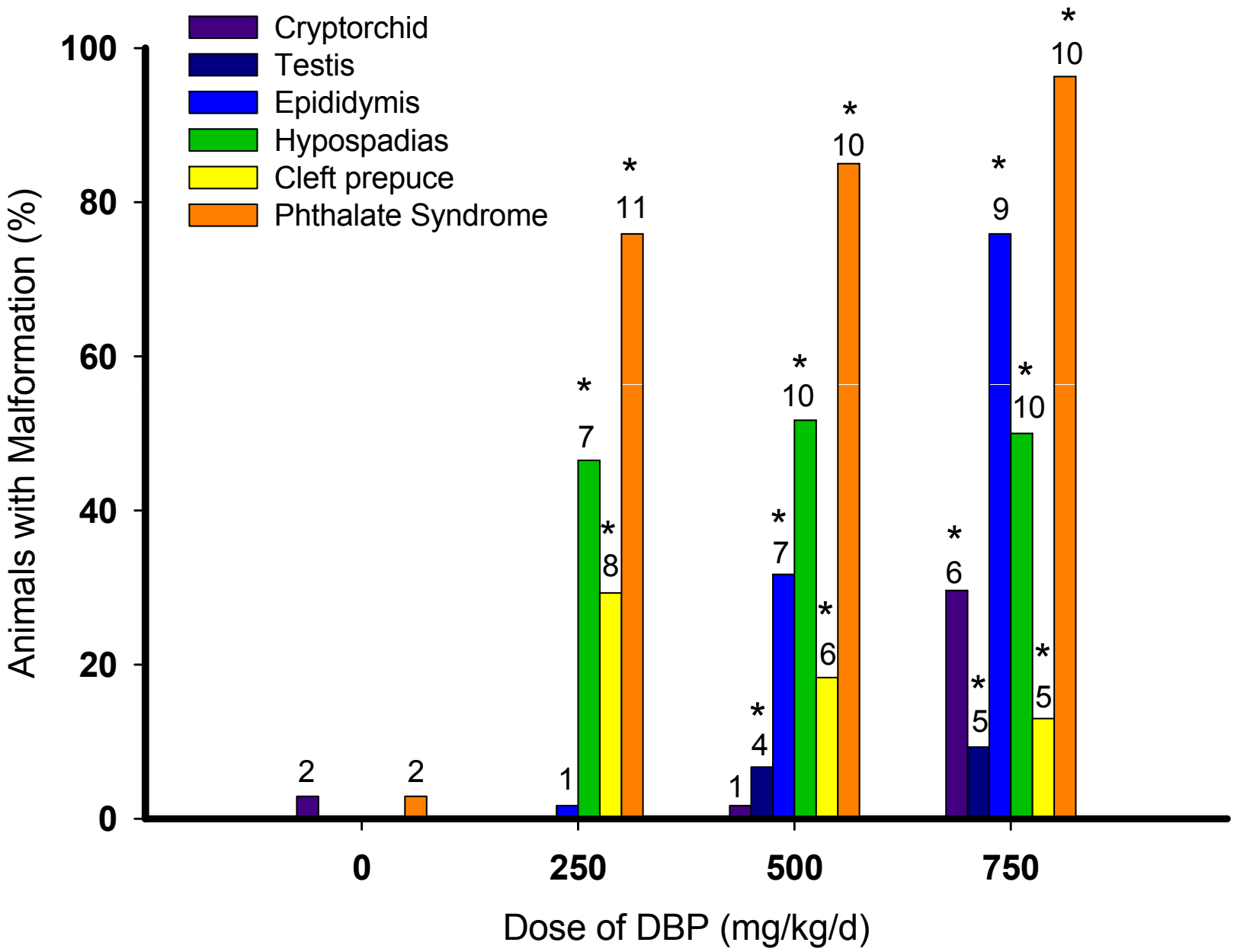
DBP dose-response: GD 12-21 rat studies

- <50 mg/kg/d – no phenotypic changes, some ↓ in fetal testicular gene expression.
- ≥50 mg/kg/d - ↓fetal testicular T, ↓gene expression, no phenotypic changes.
- ≥100 mg/kg/d - ↓AGD, ↑areolae retention, testis pathology.
- ≥250 mg/kg/d – reproductive tract malformations.
- ≥500 mg/kg/d – Leydig cell tumors.

Critical Developmental Window

- Series of experiments with 1, 2 or 3 daily doses of DBP at different times from GD 14 – 20.
- Specific malformations can be produced (at low incidence) after a single dose.
- However, the complete "Phthalate syndrome" only noted after 3 doses.
- Critical period is **before** the earliest genomic measurements conducted to date.
- Corresponds with the recently published "male programming window".

Effect of DBP over 3 days (GD15-17) on Reproductive Tract Malformations



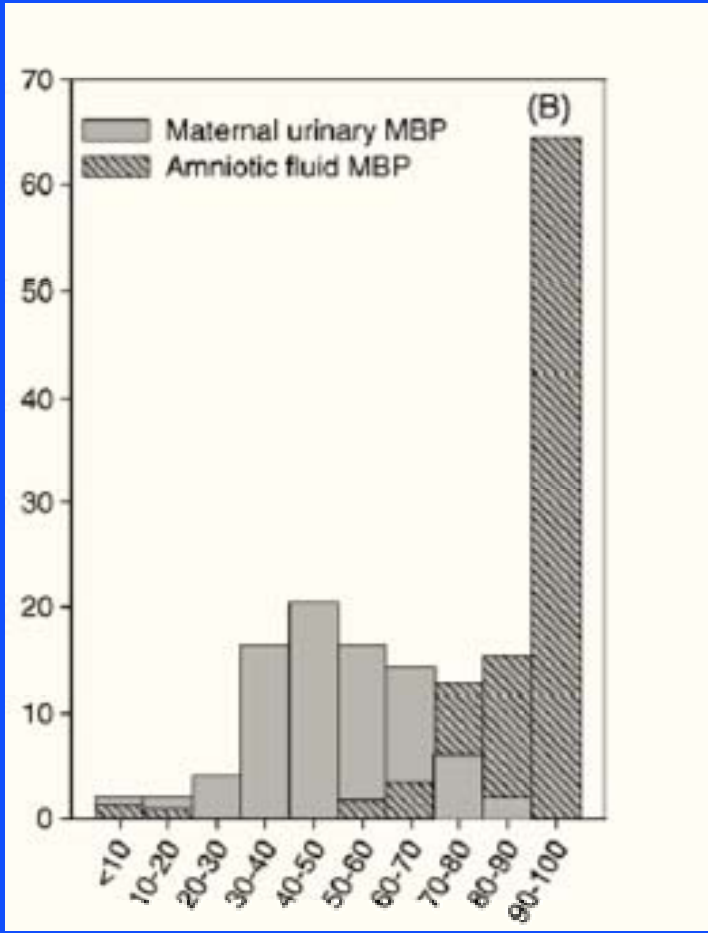
* P<0.05 (Fisher's Exact)

Numbers over bars indicate litters responding

Phthalates and Male Reproductive Development (MOA)

- Inhibition of testosterone production
  - Effects on androgen-reproductive tract development (eg epididymis, hypospadias)
- Inhibition of insl3 production
  - Malformations of the gubernaculum
  - Cryptorchidism (abdominal)
- Disturbance in gonocyte development and differentiation
  - Multinucleated cells
  - Inappropriate, age-dependent gene expression

Free MBP found in Rat Amniotic Fluid (GD 18), unlike Maternal Urine



- Critical window for induction of RT malformations. Age dependent fetal differences for UDPGT.
- DBP @ 100 mg/kg/d in rats gave an amniotic fluid mean concentration of 1400 ng MBP/mL.
- Maximum Human amniotic fluid level found of **total** MBP (54 pregnant women from general population) = 263.9 ng/mL; MOE 5.3.

Silva *et al (2004)* Bull Environ Contam Tox 72: 1226-31
Calafat *et al* (2006) *Toxicology* 217:22-30

Conclusions

- DBP (and other active phthalate esters) are antiandrogens that disrupt rat reproductive development via a disturbance in androgen signaling in the fetal testis and reproductive tract, rather than directly through the Androgen Receptor.
- DBP also inhibits *insl3* (cryptorchidism) and *c-kit* and Stem Cell Factor (multinucleate gonocytes?).
- Structure-activity relationships for reproductive toxicity are paralleled by changes in fetal testosterone production and gene expression.
- The critical developmental window for the changes in the rat is GD 15-17. Coincides with the “male programming window” for this species.
- Unlikely that PPARα has a significant role in the rat developmental effects.
- The effects in rats parallel the TDS reported for humans.

Potential role of PPARα in testis effects

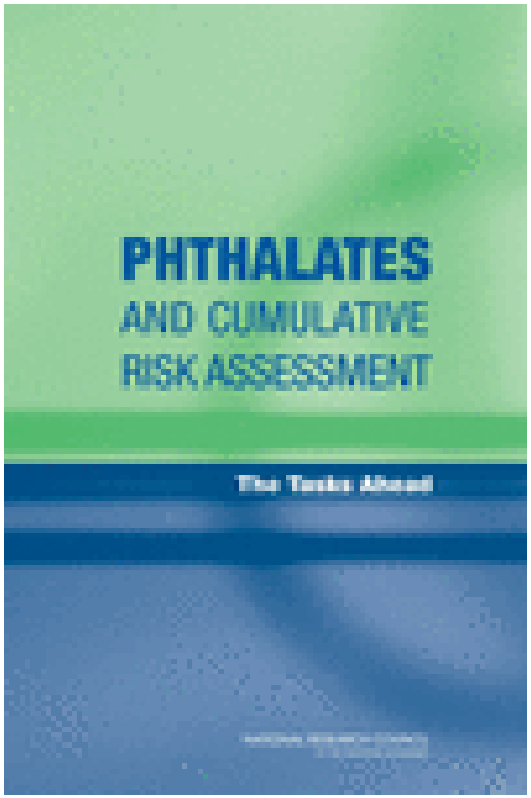
- Some active phthalates (monoesters and diesters) are weak PPARα ligands (in liver).
- They do show differential binding eg MEHP > MBP that is not reflective of potency in producing effects on the phthalate syndrome (or reductions in testosterone etc).
- Other, stronger, PPARα agonists have not reported reproductive tract malformation induction.
  - Was this specifically looked for, could they detect it?
  - For chemicals, we do have multigens that have not indicated these effects.
- Guinea pig data. A non-responsive species for the induction of peroxisome proliferation by phthalates (and other PPARα-ligands). Yet was as responsive as the rat to the juvenile testicular effects of DEHP.

Acknowledgements

- **Post Doctoral Fellows**
  - Eve Mylchreest
  - Barry McIntyre
  - Norm Barlow
  - Katie Turner
  - Chris Bowman
  - Christina Carruthers

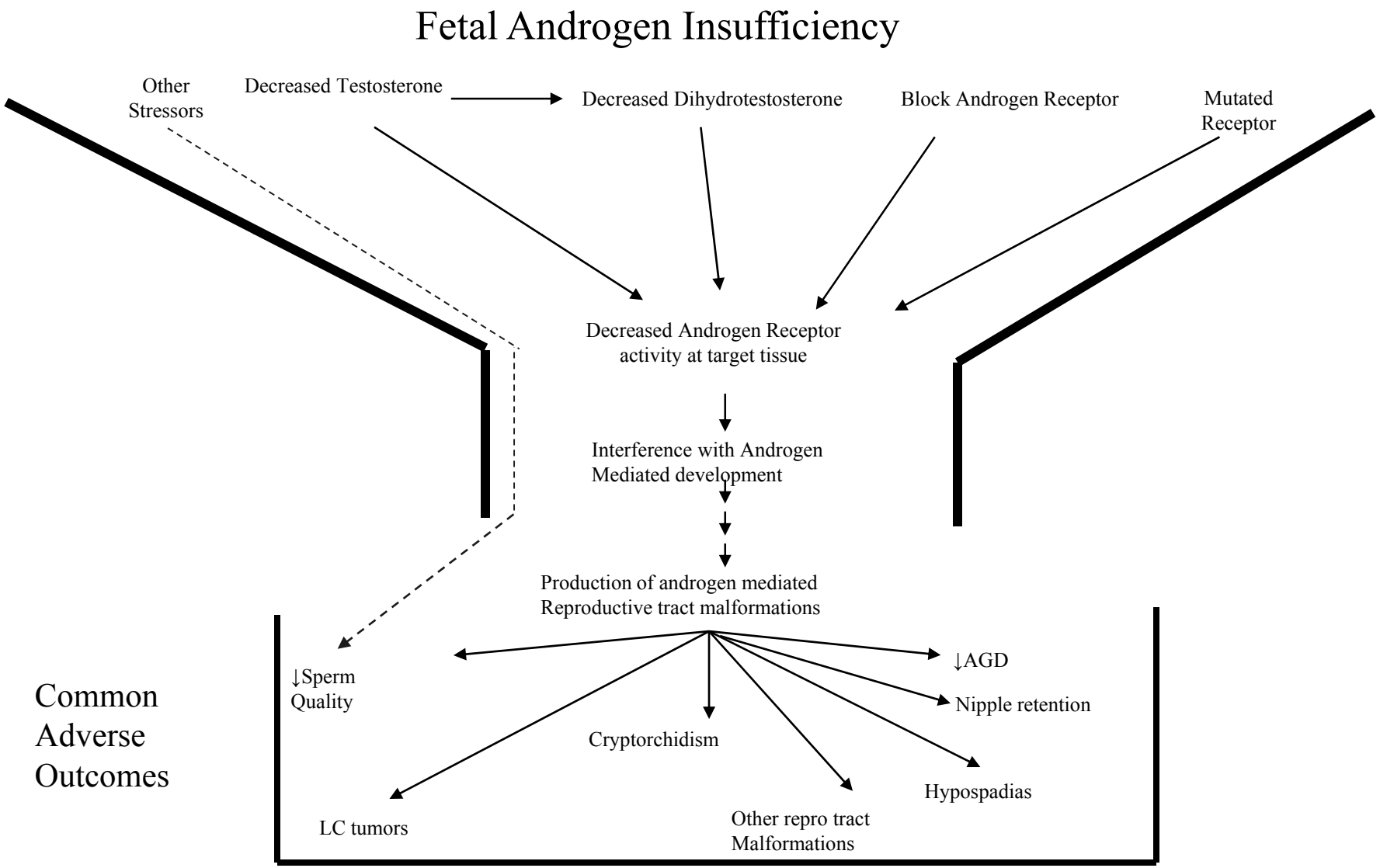
- **Collaborators**
- Kevin Gaido – CIIT (now FDA)
- Richard Sharpe
  -MRC HRSU, Edinburgh
- Earl Gray - EPA

NAS/ NRC report- December 2008



- Should a cumulative risk assessment be conducted?
- What approaches could be adopted?
- Empirical data on chemical effects on reproductive development via impairment of androgen action indicated that dose addition would model data best, even when agents act through different modes of action to produce a common adverse outcome (male reproductive malformations).
- Focus for inclusion of agents in any cumulative risk assessment was not on specific MOA's, but agents that can produce common adverse outcomes.
- "The committee concludes that it is plausible and warranted to extend cumulative risk assessment to include chemicals associated with common adverse outcomes as exemplified in this report by inclusion of other antiandrogenic chemicals with phthalates…. Not to do so would significantly underestimate potential human risk."

Phthalates and Cumulative Risk, NRC 2008



Cumulative Risk and Mixtures

- For “real world” mixtures, difficult for a Regulatory Agency to ask for mixture testing.
  - How does one ask several registrants to test the effects of multiple fungicides used on single crops?
  - Issue of Pharmaceuticals in the Environment (eg in ground or surface waters) acting together.
- While HTS and high content, low throughput screens may provide data on MOA and molecular target, they are unlikely to provide information on common adverse outcomes in the near future.
- “Adverse effect: Change in morphology, physiology, growth, development or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of a capacity to compensate for additional stress, or an increase in susceptibility to other external influences.”
  - Renwick et al (2003) Fd Chem Tox 41: 1211-1271