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HAMNER INSTITUTES FOR HEALTH SCIENCES

DiNP Dose-Response Studies: Gestation PK and Developmental Effects Postnatal Effects

Final Project Summary October 2011

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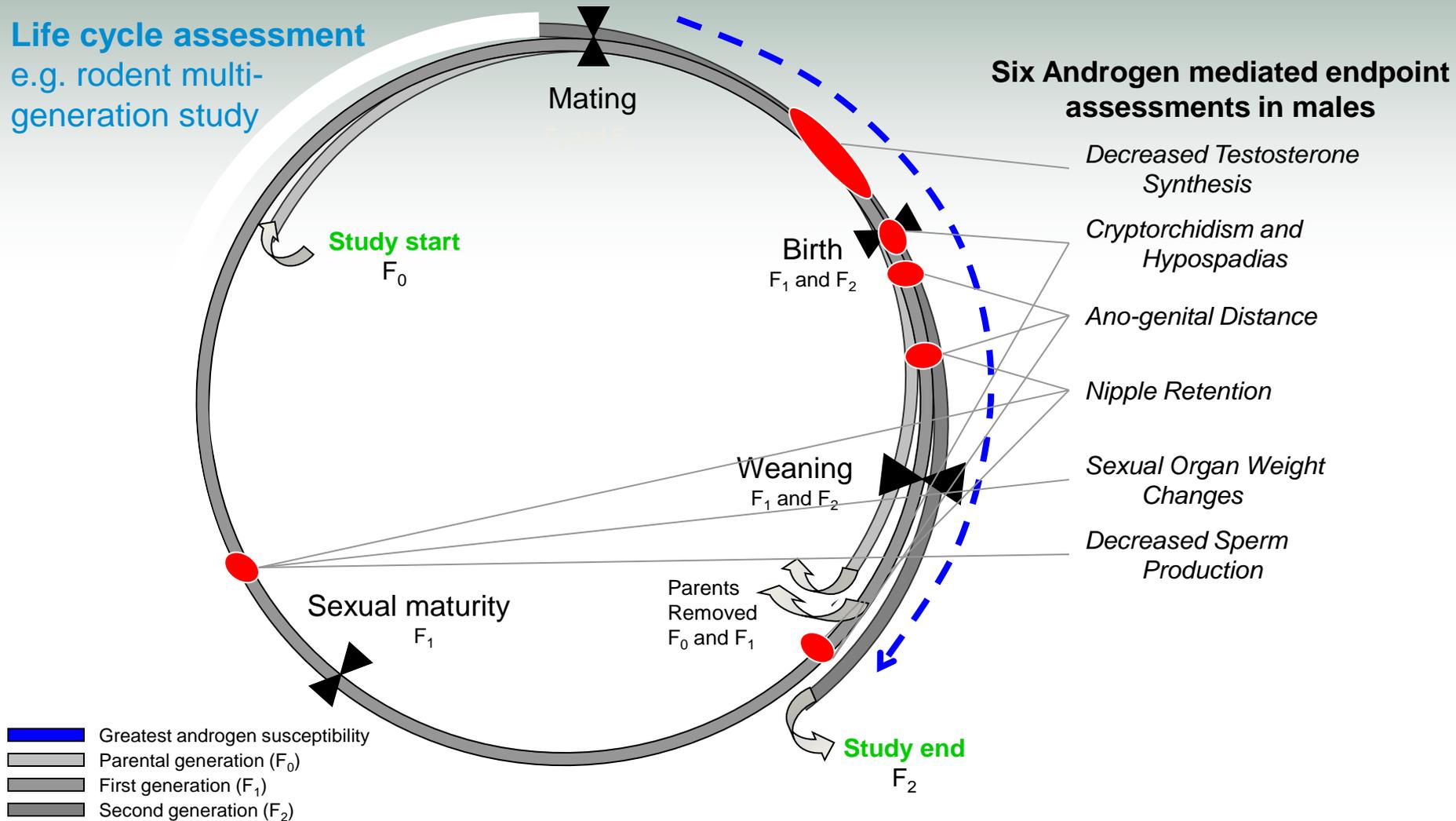
Research Investigator

Institute for Chemical Safety Sciences

Funding provided by ExxonMobil Chemical Company

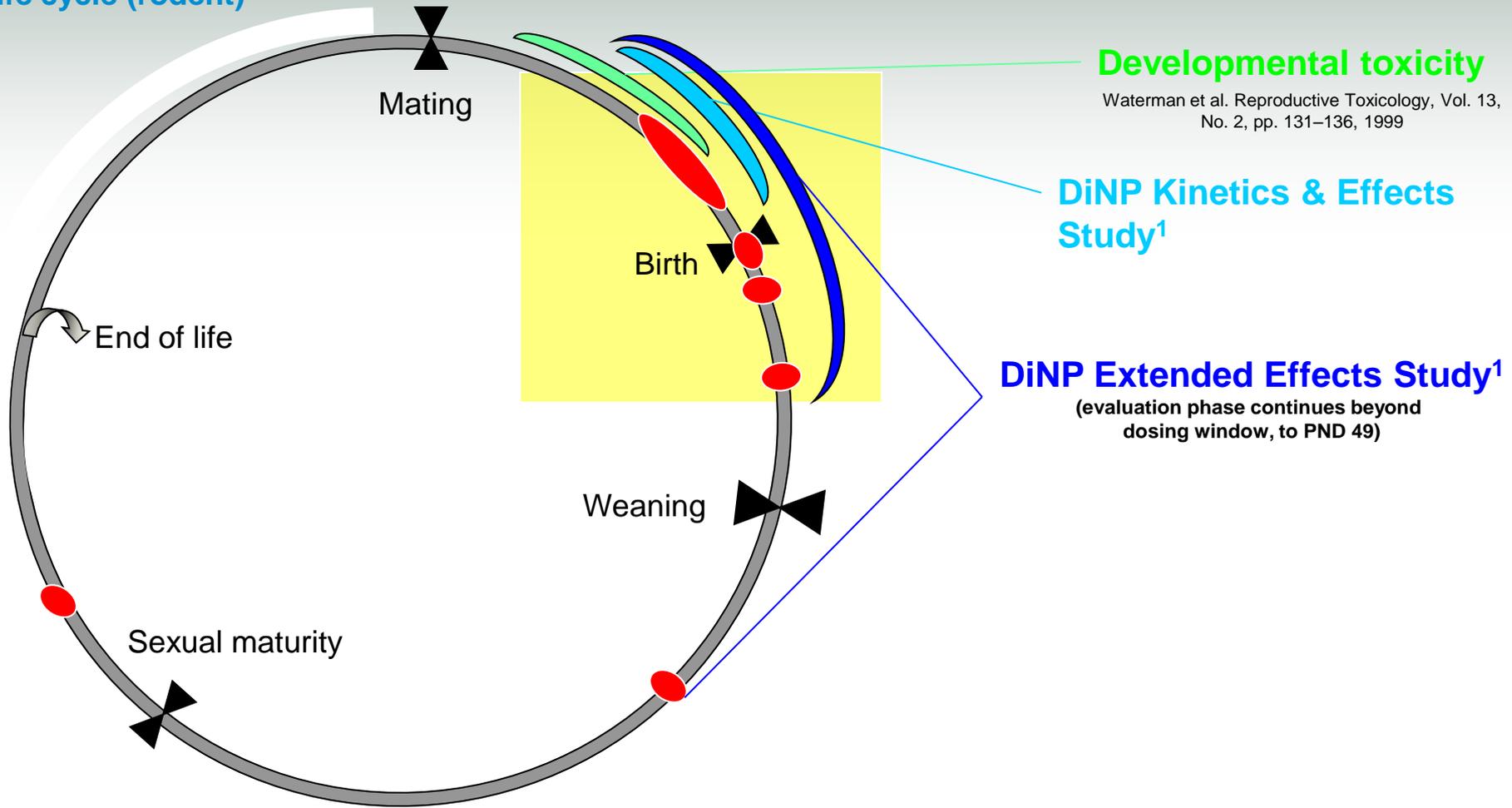
Assessing endocrine susceptibility

Life cycle assessment
e.g. rodent multi-generation study



DiNP studies cover window of susceptibility

life cycle (rodent)



Developmental toxicity

Waterman et al. Reproductive Toxicology, Vol. 13, No. 2, pp. 131–136, 1999

DiNP Kinetics & Effects Study¹

DiNP Extended Effects Study¹

(evaluation phase continues beyond dosing window, to PND 49)

● = timepoint to assess androgen endpoint

■ = window of susceptibility

Gestation Study Design

- **Objective:** comprehensively evaluate effect of fetal exposure to DiNP on the developing male rat reproductive system, determine DiNP kinetics in maternal and fetal compartments and to develop a physiologically based pharmacokinetic model
- DiNP exposure
 - Oral gavage
 - GD 12 – 19
 - 0, 50, 250, and 750 mg/kg/day
 - Time-points: 0.5, 1, 2, 6, 12, 24 hrs after final dose
- Animals
 - 144 pregnant Sprague-Dawley rats (Charles River)
 - ~ 1,700 pups
 - n = 4 dams/time-point/dose (kinetic data)
 - n = 8 dams/time-point/dose (effect data)
- Developmental Endpoints
 - Hormone
 - Testes testosterone
 - Morphology
 - AGD, testis histopathology

Gestation Study

1) Developmental Effects

GD 19, 20; 2hr, 24 hr post-dose

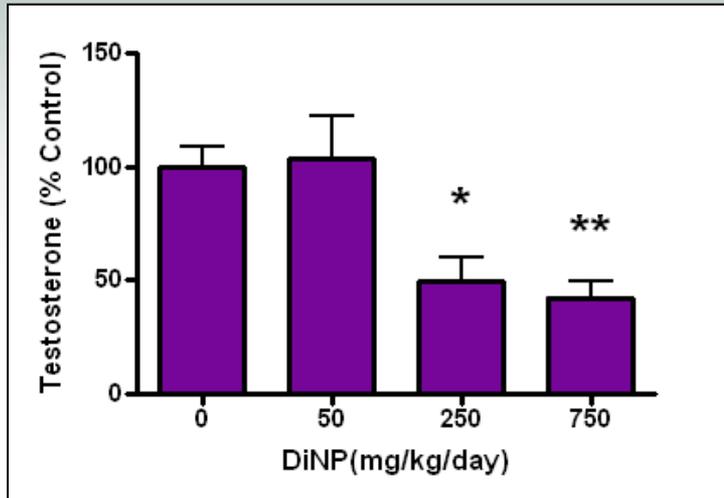
2) Pharmacokinetics

GD 19; 0.5 – 24 hr post-dose

3) PBPK Model for maternal and fetal dosimetry

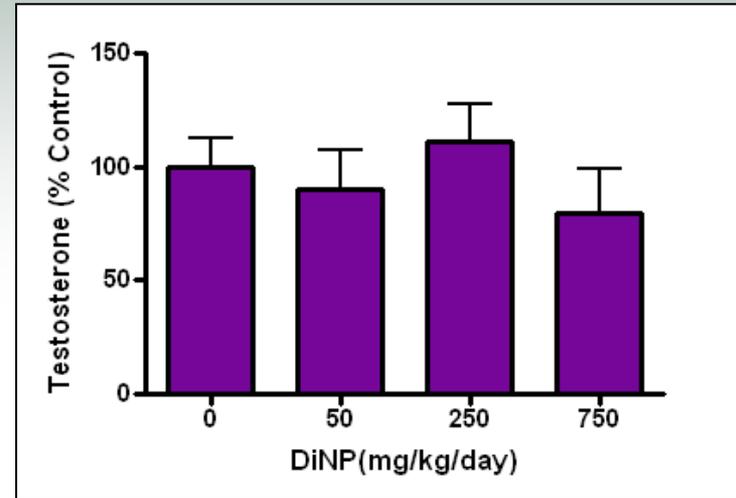
Developmental Effects- Testosterone

2 hr post-dose



* Versus concurrent controls

24 hr post-dose



* Versus concurrent controls

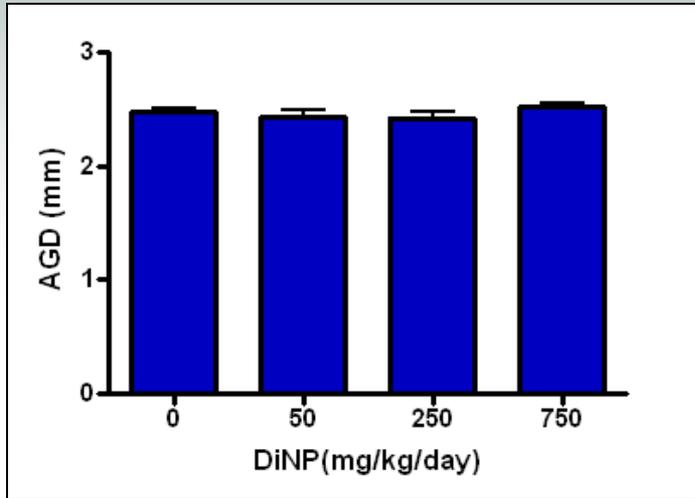
- Clear no-observed effect level for DiNP-induced decrease in testosterone at 50 mg/kg/day
- Calculated ED₅₀
 - DiNP = 389 mg/kg/day
 - DBP = 39 mg/kg/day¹
 - DEHP = 100 mg/kg/day²

¹DBP data from Mylchreest et al., 1999 (PND 2)

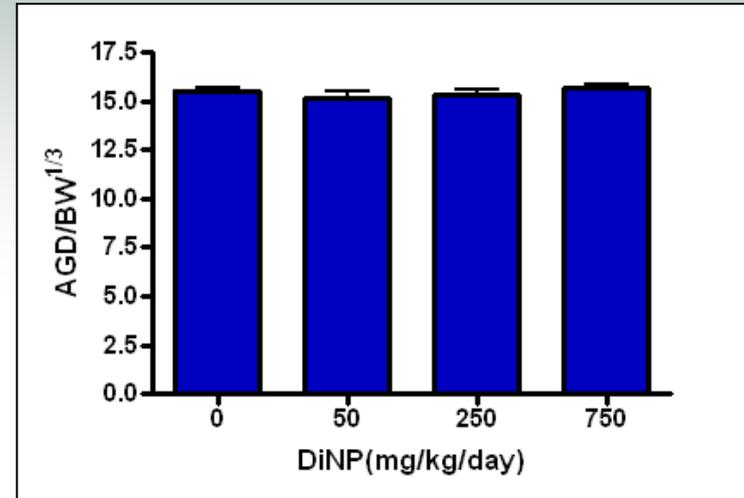
²DEHP data from Borch et al., 2004; Gray et al., 2009 (PND 2)

Developmental Effects- Anogenital Distance (AGD)

Absolute AGD



Scaled AGD



- No change in absolute or scaled AGD up to 750 mg/kg/day DiNP

•NOEL

- DiNP = >750 mg/kg/day
- DBP = 100 mg/kg/day¹
- DEHP = 100 mg/kg/day²

•LOEL

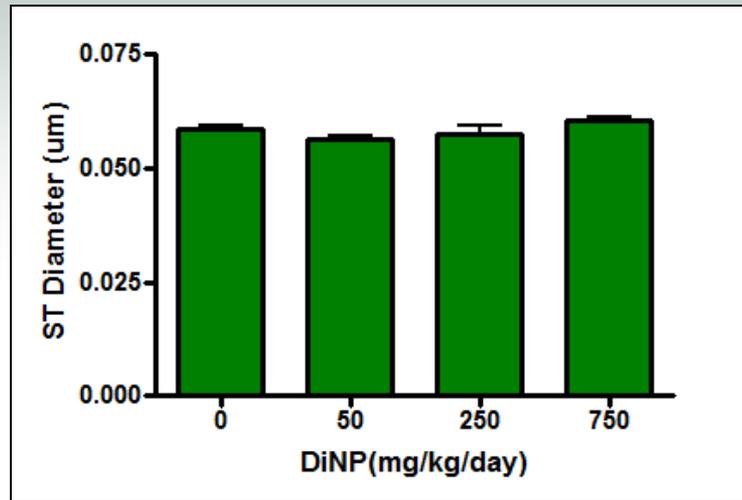
- DiNP = no LOEL identified
- DBP = 250 mg/kg/day¹
- DEHP = 300 mg/kg/day²

¹DBP data from Mylchreest et al., 1999 (PND 2)

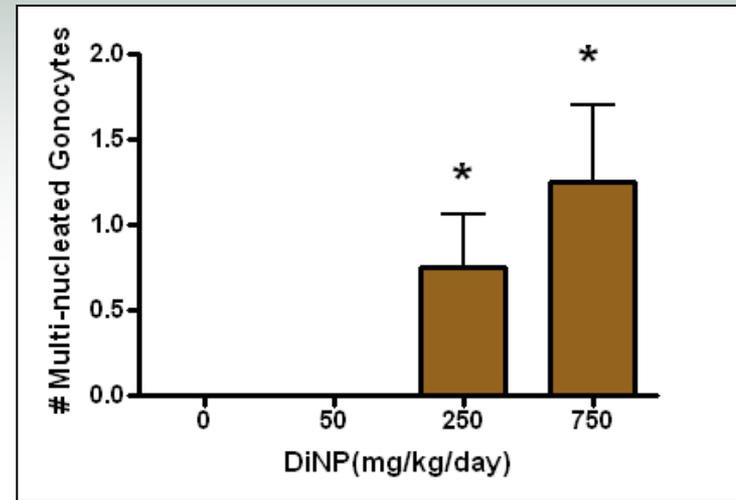
²DEHP data from Gray et al., 2009 (PND 2)

Developmental Effects- Testis Histopathology

Seminiferous Tubule (ST) Diameter^a



Multinucleated Gonocytes^b



- No change in ST diameter
- ST diameter
 - NOEL DiNP = >750 mg/kg/day
 - NOEL DBP = 30 mg/kg/day
 - LOEL DBP = 50 mg/kg/day

- Increase in #MNGs/section at 250 and 750 mg/kg/day DiNP
 - Unclear biological significance, although not testosterone dependent
- Multinucleated Gonocytes
 - NOEL DiNP = 50 mg/kg/day
 - LOEL DiNP = 250 mg/kg/day
 - NOEL DBP = 50 mg/kg/day
 - LOEL DBP = 100 mg/kg/day

^aEvaluated quantitatively using ImagePro Software at The Hamner Institutes

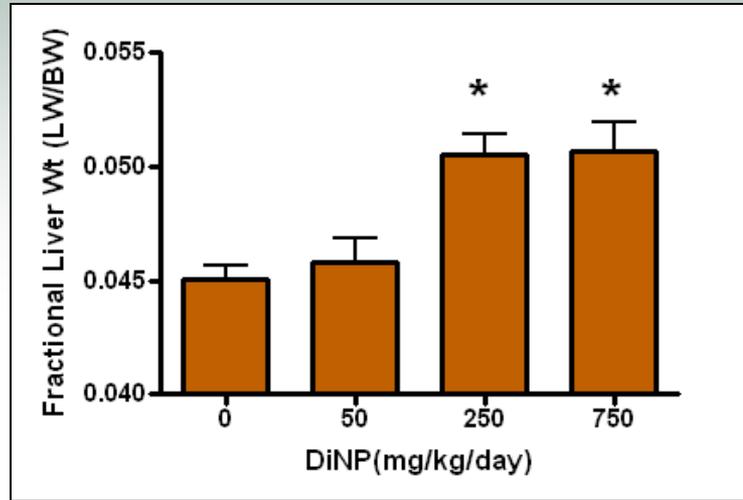
^bEvaluated by Gabrielle Willson, Experimental Pathology Laboratories, Inc.

Developmental Effects- Testis Histopathology*

DiNP Dose (mg/kg/day)	Control	50	250	750
Number of animals examined	27	8	8	8
# Animals with MNGs	0	0	2	6*
# Animals with large Leydig cell aggregates	2	3	1	7*
# Animals with increased number of gonocytes	0	0	0	2

- Statistically significant increase in the number of animals with multinucleated gonocytes (MNGs) with 750 mg/kg/day
 - based on mouse studies, effect not considered testosterone dependent
- Statistically significant increase in the number of animals with large leydig cell aggregates with 750 mg/kg/day DiNP

PPAR Effects- Fractional Liver Weight (Maternal Rat)



- As expected, increase in fractional liver weight observed with 250 and 750 mg/kg/day DiNP likely due to peroxisome proliferation¹

- NOEL

- DiNP = 50 mg/kg/day
- DBP = 200 mg/kg/day²
- DEHP = 50 mg/kg/day²

- LOEL

- DiNP = 250 mg/kg/day
- DBP = 1000 mg/kg/day¹
- DEHP = 200 mg/kg/day¹

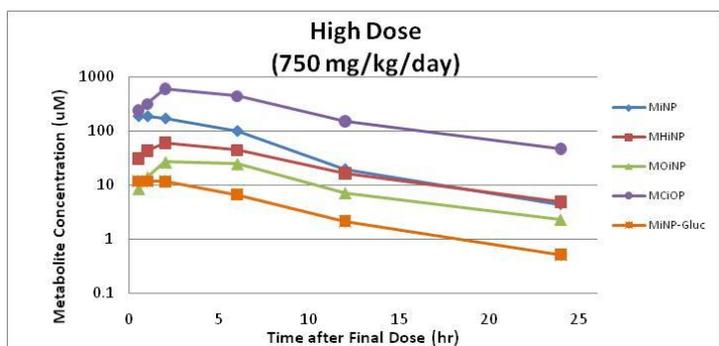
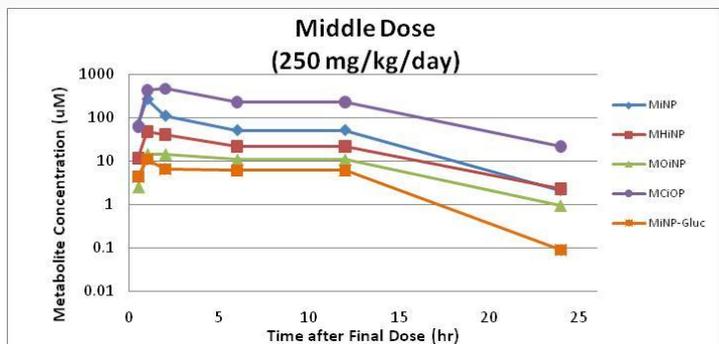
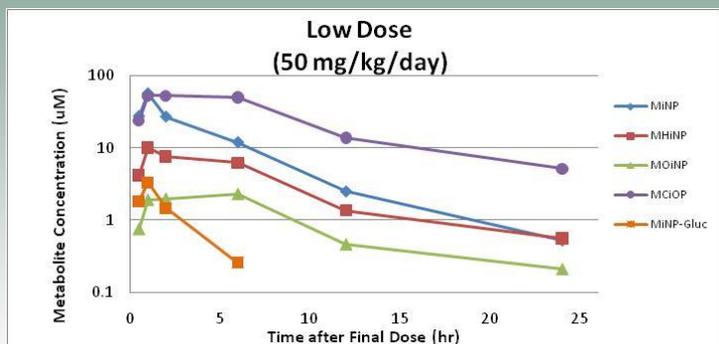
¹Mode of action (i.e. activation of peroxisome proliferator activated receptor alpha (PPAR α)) in development of rodent liver tumors has been characterized and demonstrated not active in humans

²DBP and DEHP data from Seo et al., 2004 (male SD)

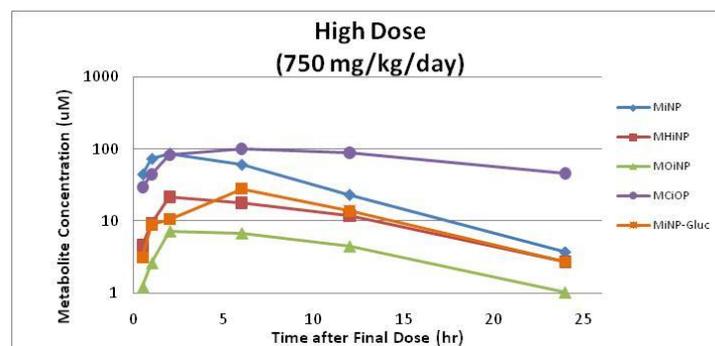
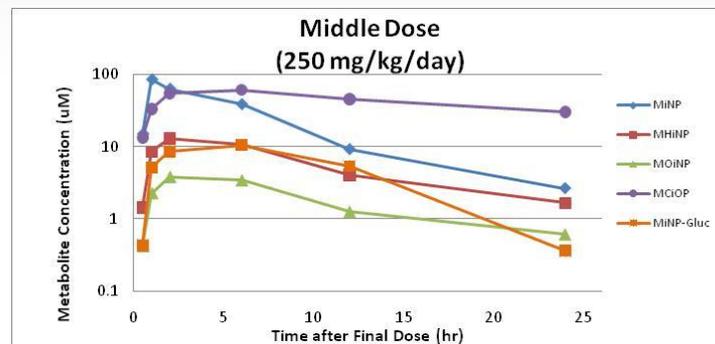
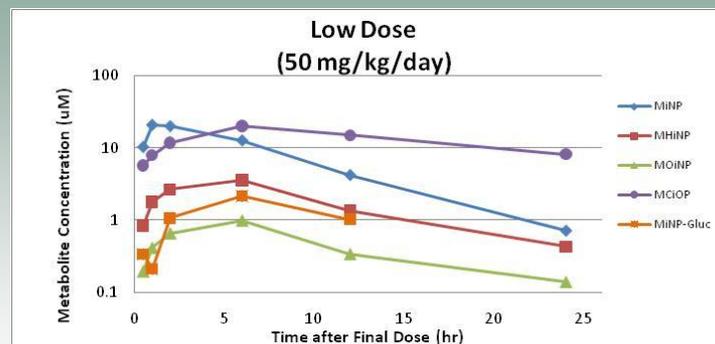
Gestation Study – DiNP Pharmacokinetics

- Preparation of MiNP-glucuronide standard
 - Glucuronide conjugation performed using human liver microsomes
 - Isolation of glucuronide by liquid chromatography
 - Confirmation of MiNP-G by NMR spectroscopy
 - Measure MiNP-G concentration by HPLC-UV/Vis
- Metabolite Analysis
 - LC-MS/MS
 - Atmospheric pressure ionization
 - Selective ion monitoring/product ion identification
 - Quantitation by isotope dilution, using ^{13}C -internal standards
- Tissues evaluated
 - Maternal plasma
 - Pup plasma
 - Urine
 - *Maternal liver, placenta*
 - *Fetal testes, amniotic fluid*

Maternal Plasma



Fetal Plasma

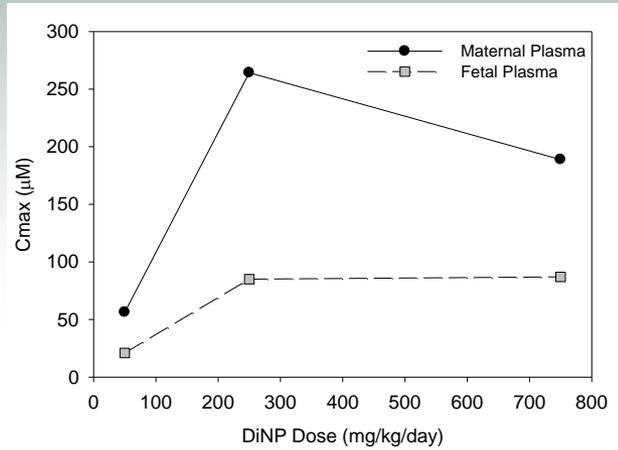


- Concentration of metabolites in maternal plasma: MfOP > MfNP > MHiNP > MOiNP > MfNP-G
- MfNP-G detected at all doses indicating that glucuronide-conjugation occurs in the rat

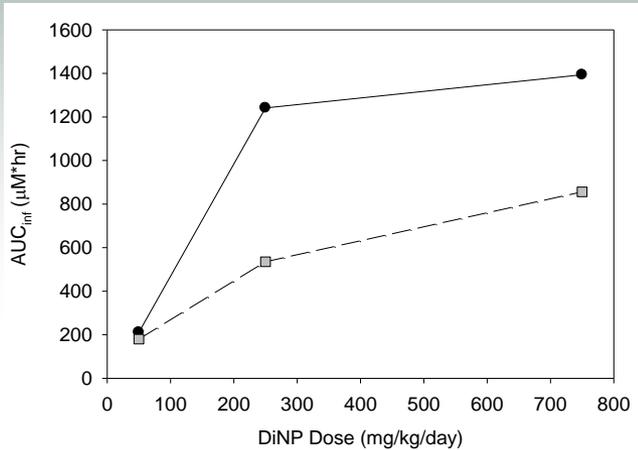
- Concentration of metabolites in fetal plasma: MfOP > MfNP > MHiNP, MOiNP, and MfNP-G
- MfNP-G generally higher than the maternal plasma

Plasma PK Analysis - MiNP

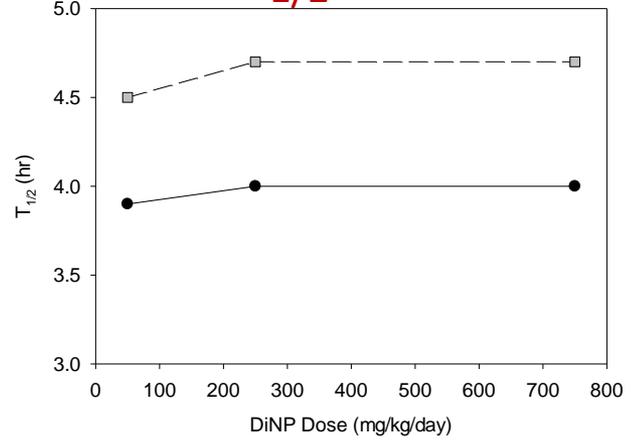
Cmax



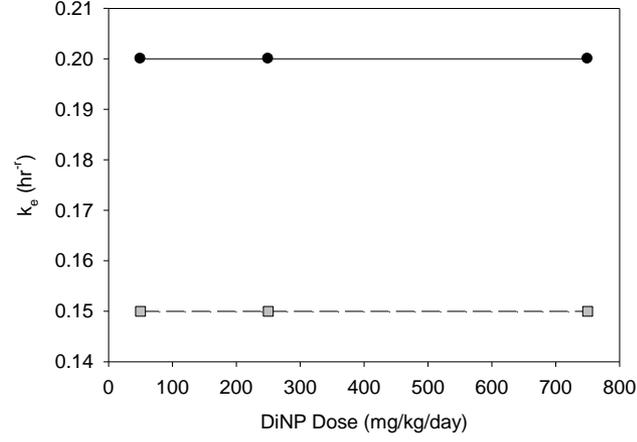
AUC



T_{1/2}

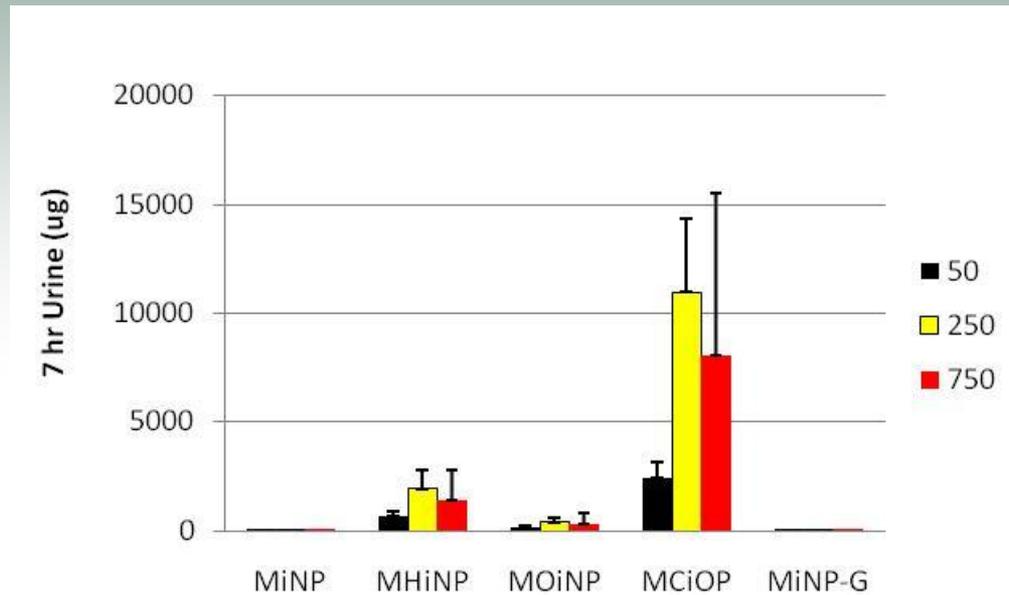


Ke



- Non-linearity in C_{MAX} and AUC, but not K_e or T_{1/2}, suggests **absorption limitation**

Maternal Urine Metabolites

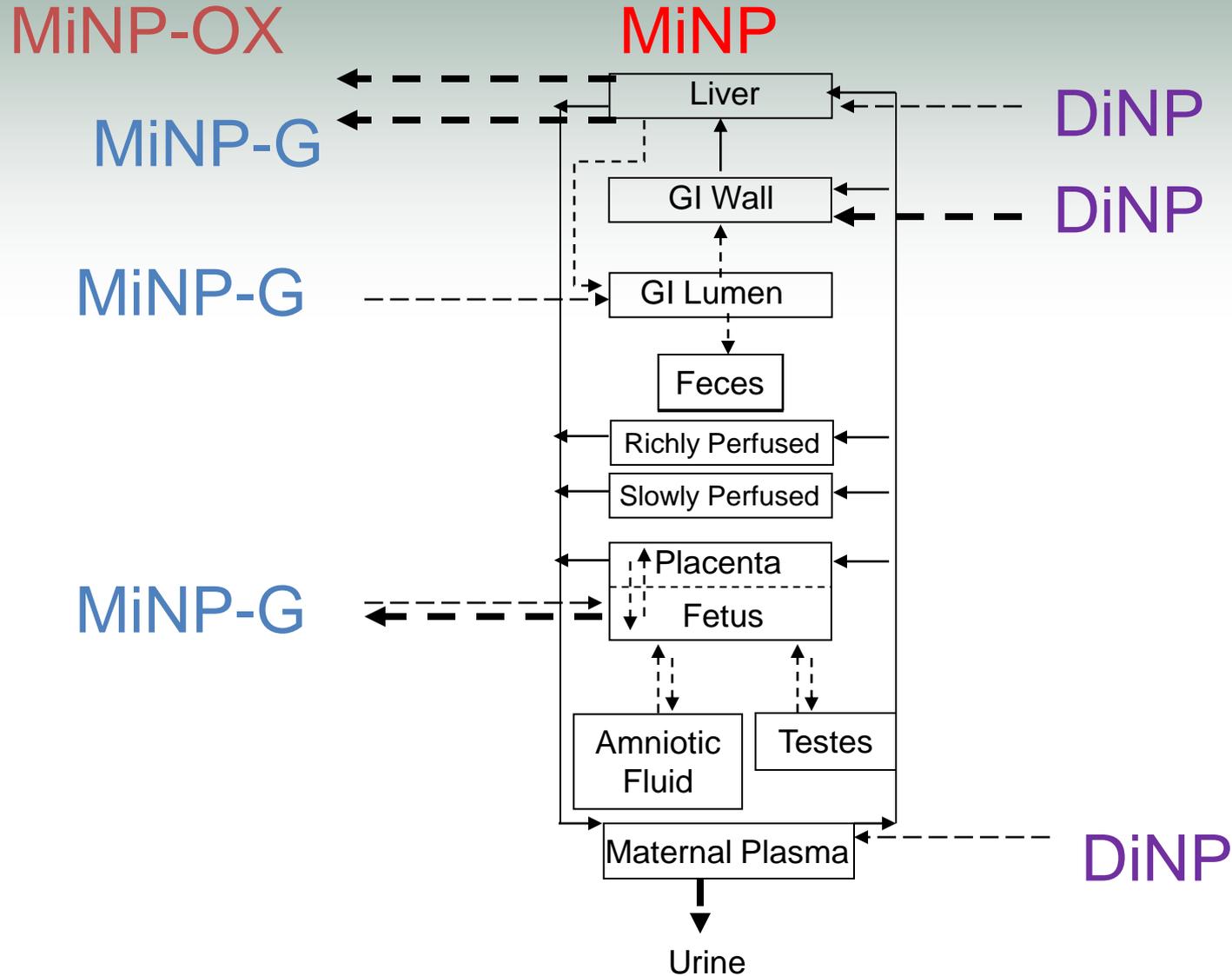


- MCIOP >> MHiNP > MOiNP >>> MiNP, MiNP-G
- MiNP and MiNP-G are present
 - Account for < 0.1% of dose.

PBPK Model for DiNP – Preliminary Analysis

- DEHP Model Structure
 - Glucuronidation turned off
 - Combined oxidative metabolites ($\text{MiNP-OX} = \text{MHiNP} + \text{MOiNP} + \text{MCiNP}$)
- First run used DEHP parameters
 - No adjustments other than molecular weight
 - Assume similar partitioning for MiNP and MiNP-OX
- Parameter adjustments for:
 - Oral absorption: water emulsion (DEHP) vs. corn oil (DiNP)
 - Placental transfer of ox metabolites (not measured with DEHP)

Phthalate PBPK Model Structure

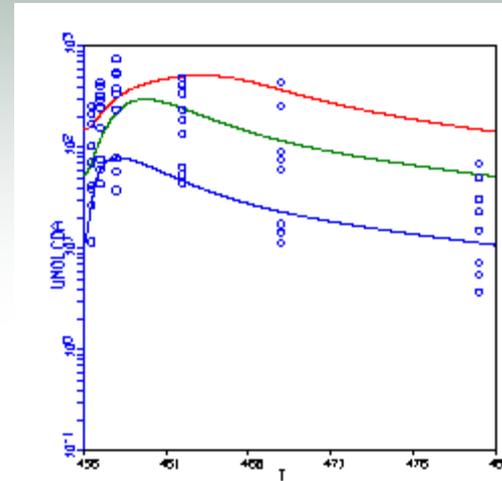
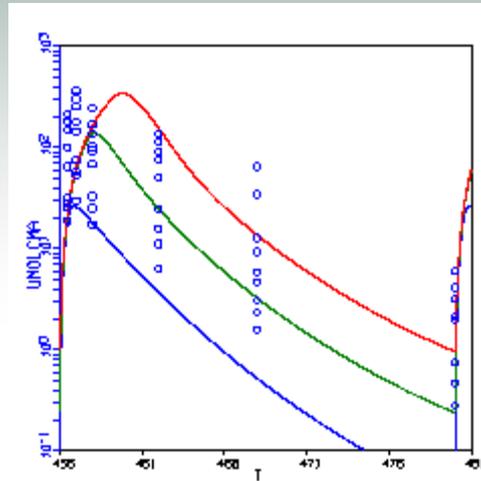


DiNP PBPK Model – Initial fits

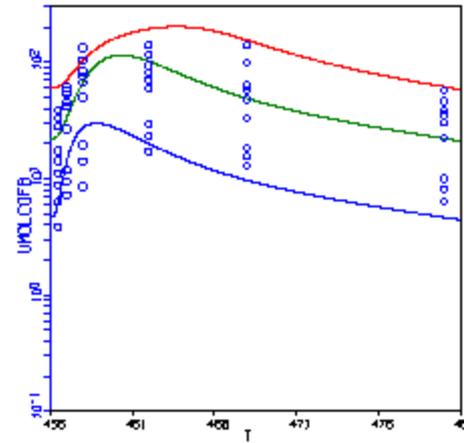
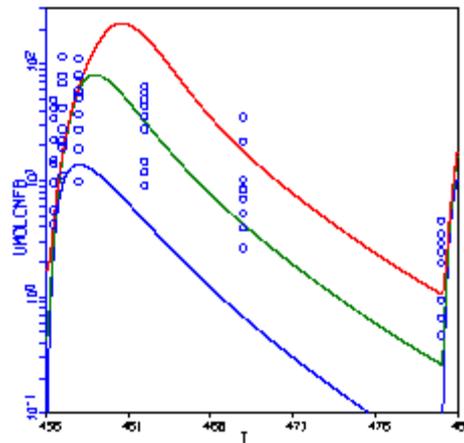
MiNP

MiNP-Ox

Maternal
Plasma



Fetal
Plasma



- Using DEHP parameters

Summary – DiNP Gestation Study

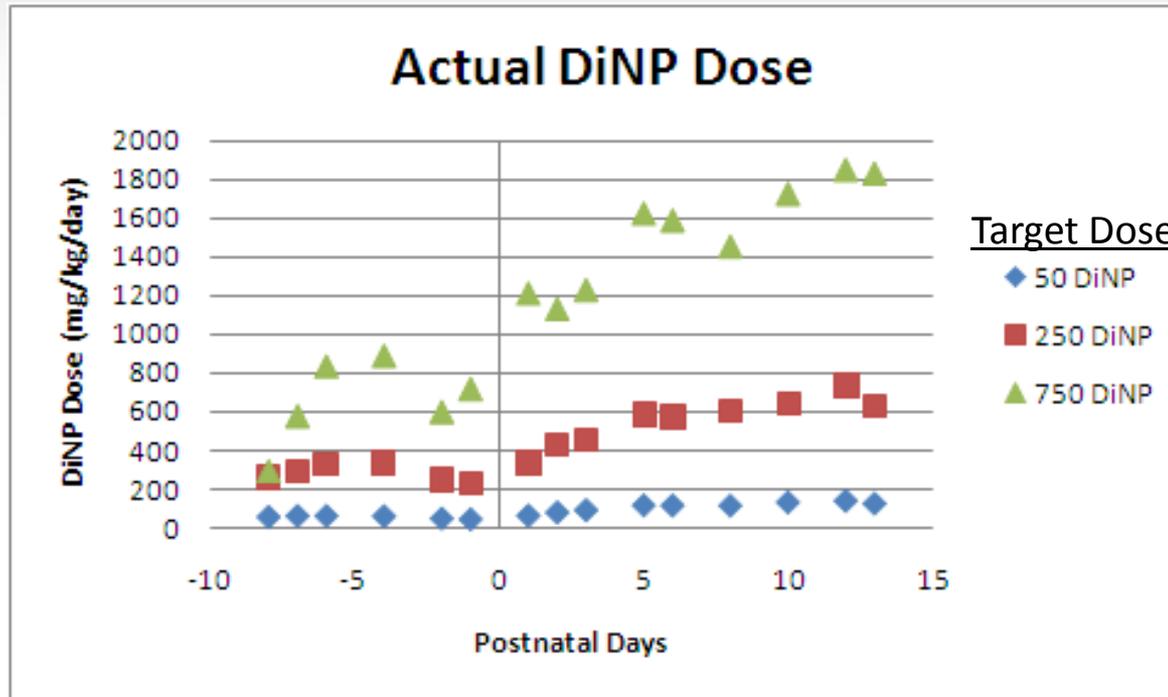
- NOEL for DiNP effects
 - 50 mg/kg/day: T inhibition, MNG, increased liver weight
 - >750 mg/kg/day: AGD, ST diameter
- DiNP metabolites are present in the fetal testes
- Apparent saturation of oral absorption at highest dose (750 mg/kg/day)
 - Evidenced by tissue metabolite data
 - Causes plateau in liver wt and T inhibition
 - Likely result of oral gavage administration
- DiNP is consistently less potent than DBP and DEHP where there is equivalent D-R data
- Similar kinetics to DEHP, indicates reduced potency of DiNP is due to **pharmacodynamic** differences

Postnatal Effects Study^A

- **Objective**: determine a NOEL for effects on the developing male rat reproductive tract for di-isononyl phthalate (DiNP).
- Study Design
 - Very large n for statistical significance: 20 – 24 litters per treatment group
 - Begin dietary dosing GD 12
 - Weigh dams and food 4x per week
 - All necropsies and observations completely BLINDED
 - PND 2: necropsy 1 male, cull to 8 pups/litter
 - PND 21: euthanize all females
 - PND 49: necropsy all males
- Endpoints
 - PND 2
 - AGD, testes testosterone, testis/epididymis histopathology
 - PND 14
 - AGD, nipple retention
 - PND 49
 - AGD, nipple retention, testes testosterone
 - Hypospadias (phallus malformation), preputial separation
 - Morphology/tissue weight of 10 reprod. tissues, liver, kidney

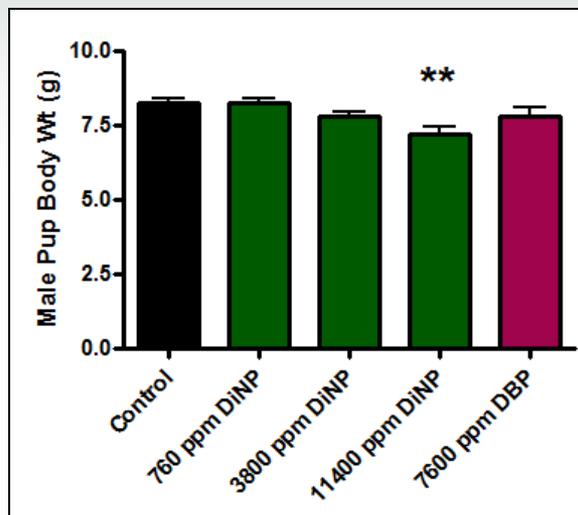
DiNP Dose

- Calculated from DiNP concentration (measured) and food consumption (measured)



Postnatal Effects Study – PND 2

Body Weight (males)

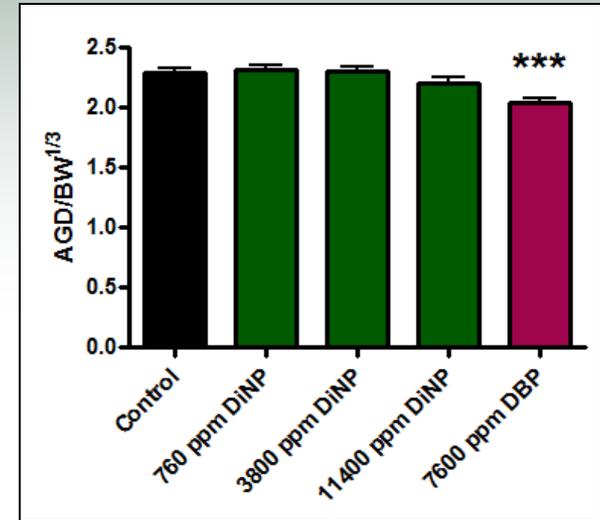


- Pup body weights were reduced in the 11400 ppm DiNP group on PND 2

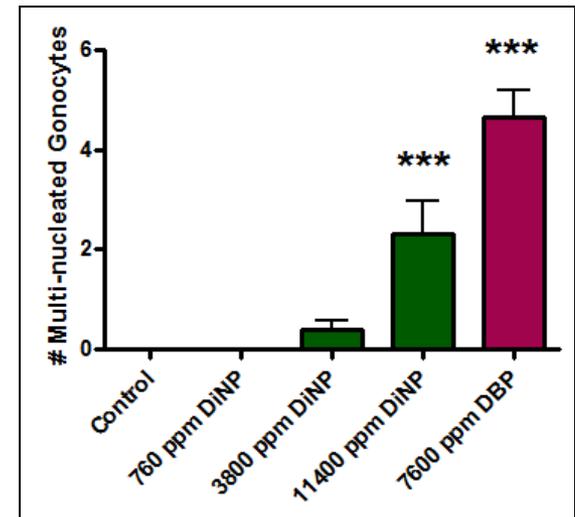
Postnatal Effects Study – PND 2

- No statistically significant change in testicular testosterone content with DiNP
- No change in absolute or scaled AGD or seminiferous tubule diameter with DiNP
 - Absolute and scaled AGD reduced with DBP
- No change in relative testes or epididymis weights
- Increase in number of multinucleated gonocytes/section
 - Incidence rate was low compared to DBP
 - Unclear biological significance, although not testosterone dependent

AGD (scaled by $BW^{1/3}$)



Multinucleated Gonocytes

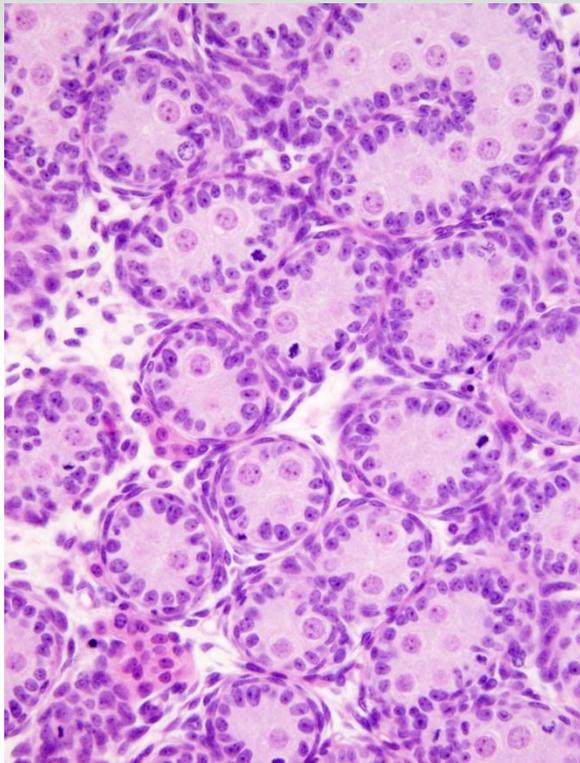


Postnatal Effects- PND 2 Testis Histopathology*

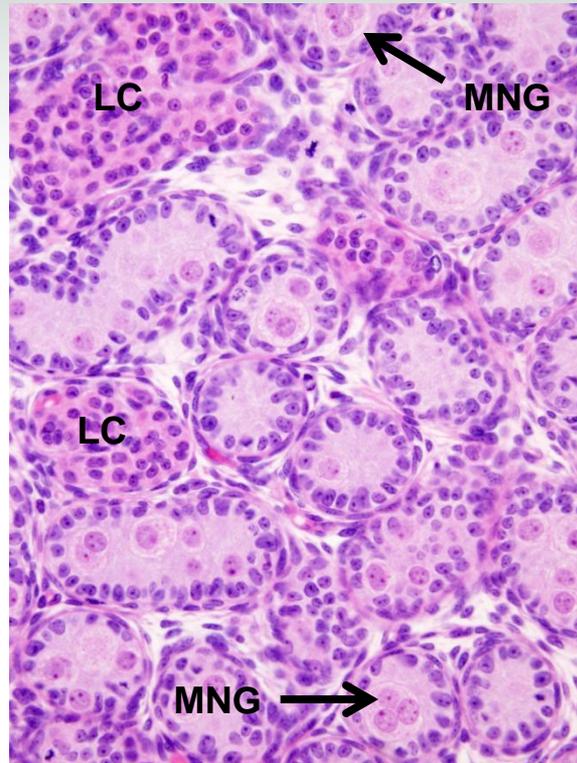
	Control	DiNP 760 ppm	DiNP 3800 ppm	DiNP 11400 ppm	DBP 7600 ppm
Number of animals examined	24	20	20	19	21
# Animals with MNGs	1	2	7*	18**	21**
# Animals with large Leydig cell aggregates	4	4	8	19**	18**
# Animals with increased number of gonocytes	0	0	0	0	5*

- Statistically significant increase in the number of animals with multinucleated gonocytes (MNGs) at 250 and 750 mg/kg/day DiNP
 - based on mouse studies, effect not considered testosterone dependent
- Statistically significant increase in the number of animals with large leydig cell aggregates at 750 mg/kg/day DiNP

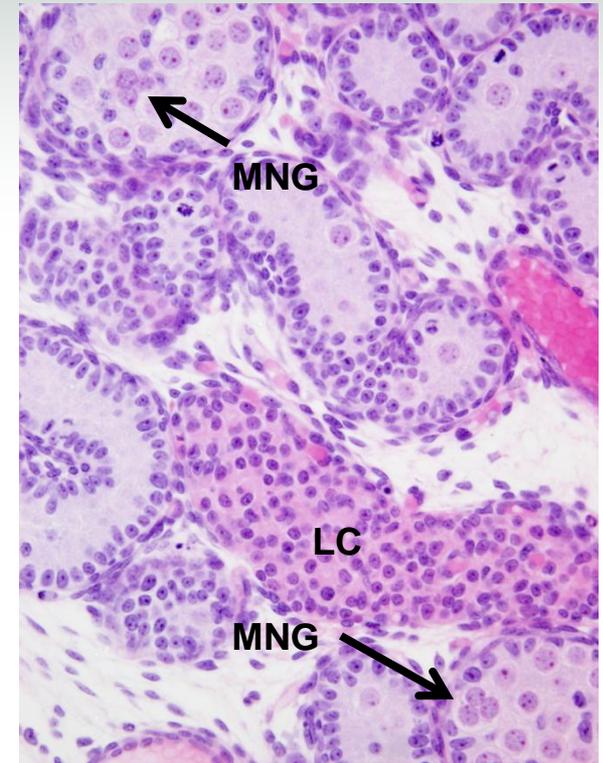
Postnatal Effects Study – Histopathology



Control



11400 ppm DiNP

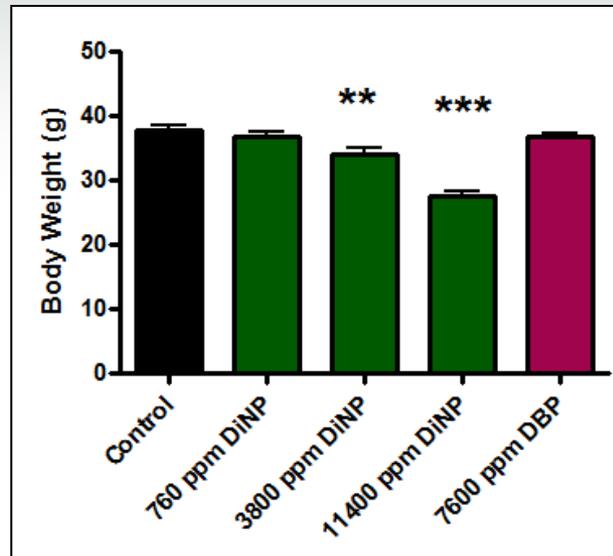


7600 ppm DBP

MNG = Multinucleated gonocytes
LC = Large Leydig cell aggregates

Postnatal Effects Study – PND 14

Body Weight (males)

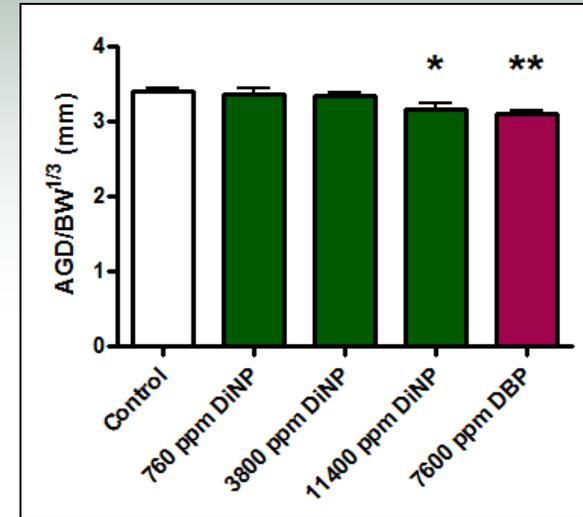


- Pup body weights were reduced in the 3800 and 11400 ppm DiNP groups on PND 14

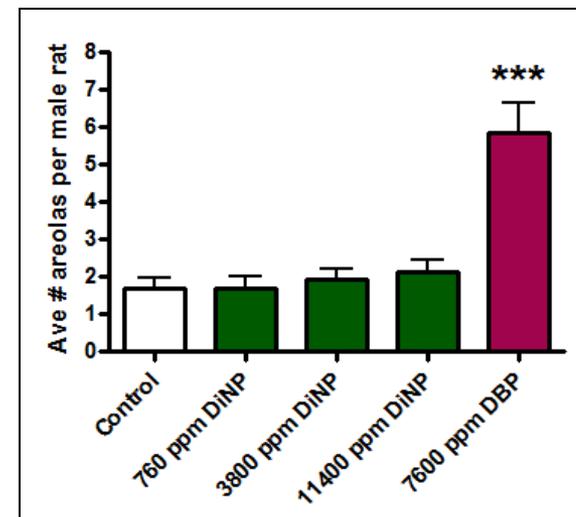
Postnatal Effects Study – PND 14^A

- Reduced AGD with 11400 ppm DiNP and DBP^A
- No increase in nipple retention with DiNP
 - Increased nipples with DBP

AGD (scaled by BW^{1/3})



Nipples/Areolae



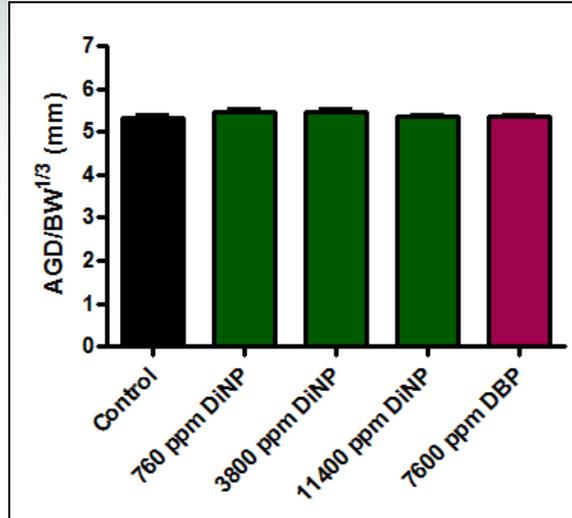
^ARevised from July 2011 slides

Mean values shown for all control ($n = 24$) and DiNP ($n = 20$) or DBP ($n = 21$) exposed litters.

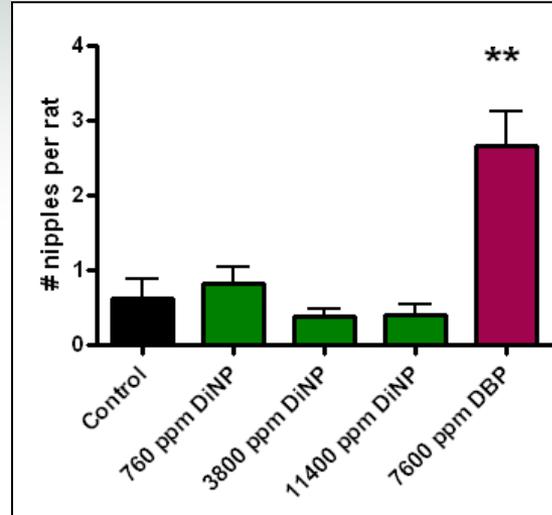
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 1-way ANOVA with Dunnett's post-test, using the litter as the statistical unit

Postnatal Effects Study – PND 49

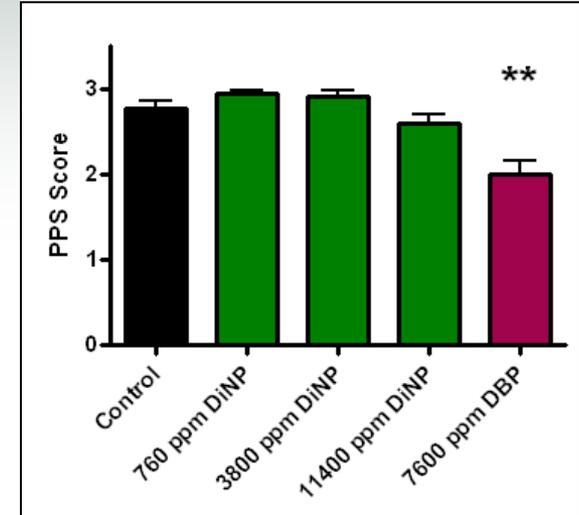
AGD (scaled by $BW^{1/3}$)



Nipple Retention



Preputial Separation



- No effect on absolute or scaled AGD for either DiNP or DBP
- No increase in nipple retention with DiNP
- clear increase observed with DBP
- No effect on preputial separation with DiNP
- decrease in preputial separation with DBP

Postnatal Effects Study – PND 49

Testis Histopathology*

	Control	DiNP 760 ppm	DiNP 3800 ppm	DiNP 11400 ppm	DBP 7600 ppm
Number examined	25	20	20	20	25
Tubular/rete dilation	1	0	0	1	4 ^a
Occasional atrophic tubules	2	1	0	1	6
Tubular dysplasia	0	0	0	0	1 ^a
Multinucleate germ cells	0	0	0	1	3

- No statistically significant histopathologic alterations of the male rat testis were seen with DiNP
- A NOEL of 11400 ppm DiNP was established for alterations in male rat testis histopathology

^aIncludes testes sampled for gross abnormalities (3 rats with tubular dilation, 1 rat with tubular dysplasia)

*Evaluated in blinded manner by Dr. Dianne Creasy, Huntingdon Life Sciences, Inc.

Postnatal Effects Study – PND 49^A

	Control	760 ppm DiNP	3800 ppm DiNP	11400 ppm DiNP	7600 ppm DBP
Body Wt (g)	299	302	296	286	305
Testes Pair Wt (% BW)	0.866	0.860	0.888	0.873	0.883
Epididymides Pair Wt (%BW)	0.141	0.146	0.145	0.142	0.134
Seminal Vesicles Wt (%BW)	0.158	0.162	0.160	0.147	0.119**
Ventral Prostate Wt (% BW)	0.082	0.085	0.080	0.078	0.070*
Glans Penis Wt (%BW)	0.034	0.030	0.032	0.032	0.030
LABC Wt (%BW)	0.205	0.202	0.198	0.191	0.168**
Cowpers Glands Wt (% BW)	0.016	0.016	0.016	0.017	0.015
Adrenals Wt (% BW)	0.014	0.015	0.015	0.017	0.015
Kidney Pair Wt (% BW)	0.888	0.888	0.914	0.893	0.851*
Liver Wt (%BW)	5.204	5.355	5.212	5.320	5.273
Ave Gubernacular Cord (mm)	4.3	4.8*	4.4	4.6	4.5

^ATable revised from July 2011 slides

Mean values shown for all control (n = 25) and DiNP (n = 20) or DBP (n = 21) exposed litters.

*p<0.05, **p<0.01, 1-way ANOVA with Dunnett's post-test, using the litter as the statistical unit.

Postnatal Effects Study – PND 49

	Control	760 ppm DiNP	3800 ppm DiNP	11400 ppm DiNP	7600 ppm DBP
Epididymal Agenesis (Incidence, total litters)	0/24	0/20	0/20	0/20	2/21
Incomplete Epididymis (Incidence, total litters)	0/24	2/20	0/20	0/20	8/21**
Flaccid Epididymis (Incidence, total litters)	2/24	2/20	4/20	3/20	7/21*
Undescended Testes (Incidence, total litters)	0/24	1/20	1/20	0/20	1/21
Atropic Testis/Epididymis (Incidence, total litters)	0/24	0/20	0/20	0/20	1/21
Mild/Slight Hypospadias (Incidence, total litters)	1/24	0/20	0/20	1/20	5/21
Exposed Os Penis (Incidence, total litters)	0/24	0/20	0/20	0/20	1/21

- DiNP did not induce significant permanent alterations in epididymal development
- A NOEL of 11400 ppm DiNP was established for alterations in epididymal histopathology

* $p < 0.05$ using nesting approach to account for incidence data in multiple pups per litter (Haseman, 1979).

Comparison of effects - DiNP Postnatal Study^A

- 500 mg/kg/day DBP

- No body weight effects

- Nipple retention
- AGD (absolute and scaled) PND 2 + 14
- Phallus development
- Epididymal development
- Preputial separation
- Weight of 4 reproductive organs

- PND 2 ST – some enlarged tubules
- PND 2 #MNG/section, large LC aggregates
 - Effects were seen to be transient (not observed at PND 49)

- ≥ 250 mg/kg/day DiNP

- PND 2 body weight (750 mg/kg)
- PND 14 body weight (≥ 250 mg/kg)

- PND 14 reduced AGD (750 mg/kg)

- No change in ST diameter
- PND 2 # MNG/section (≥ 250 mg/kg), large LC aggregates (750 mg/kg)
 - Effects were seen to be transient (not observed at PND 49)

Conclusions

- The current studies on DiNP are the most well designed, comprehensive studies available for testing the effects of DiNP on the male reproductive tract
- A clear NOEL for effects on the developing male rat reproductive tract was established for DiNP of 760 ppm (50 mg/kg/day)
- A LOEL of 3800 ppm DiNP (250 mg/kg/day) based on the significant increase in MNGs on GD 20/PND 2, testosterone reduction on GD 19, and decreased pup body weight on PND 14
 - All effects were recoverable at later time points
- No evidence for DiNP-induced effects attributed to the rat phthalate syndrome at doses up to 750 mg/kg/day using global statistical analysis
- The role of testosterone as part of the mechanism leading to each of the male reproductive effects is unclear
 - Data indicate that decreased testosterone may be necessary for the induction of some effects, but is clearly not sufficient at doses up to 750 mg DiNP/kg/day
- Although the kinetics of DiNP are similar to DEHP, the mechanism and/or potency of DiNP is different