THE 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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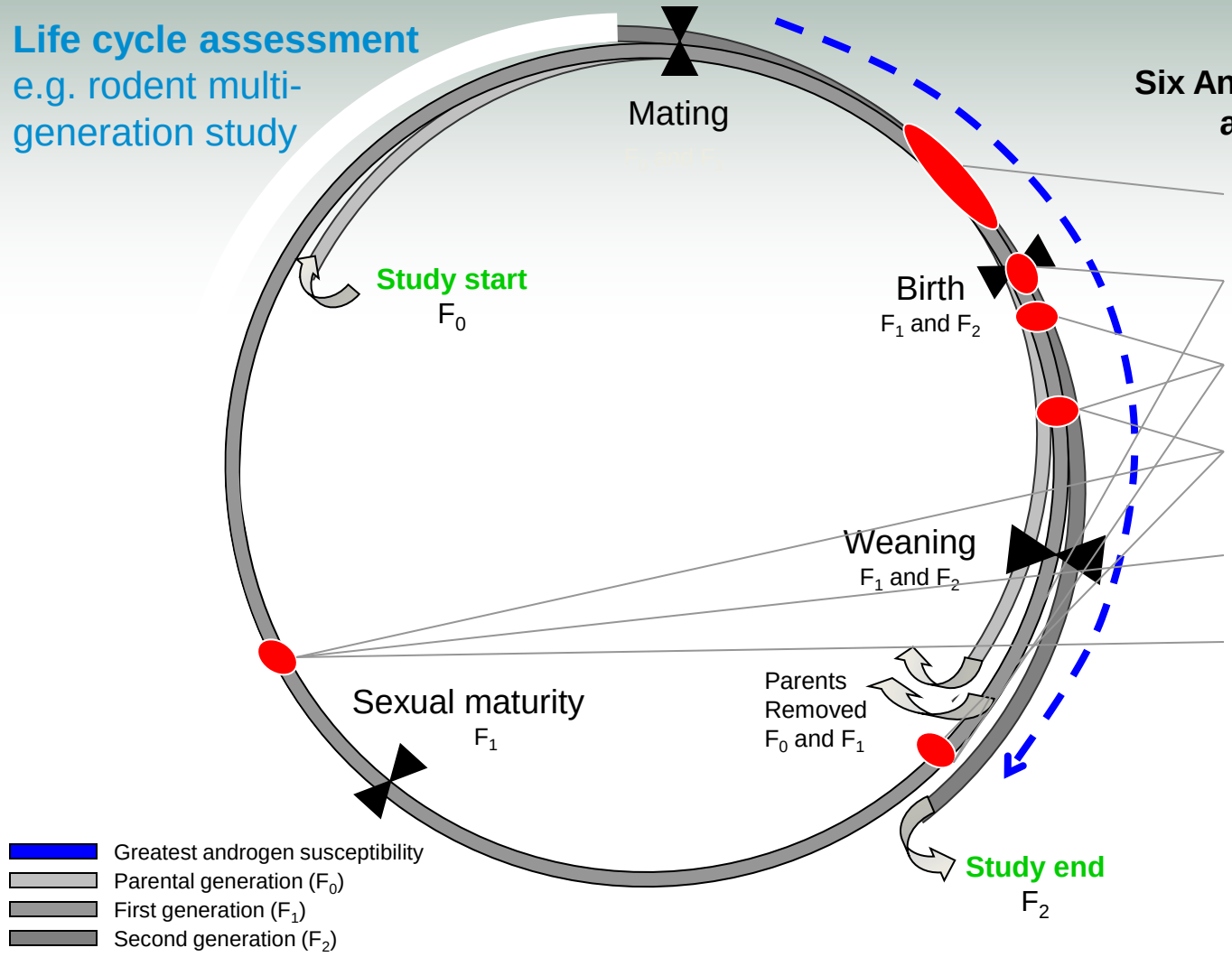
Funding provided by ExxonMobil Chemical Company

Assessing endocrine susceptibility

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

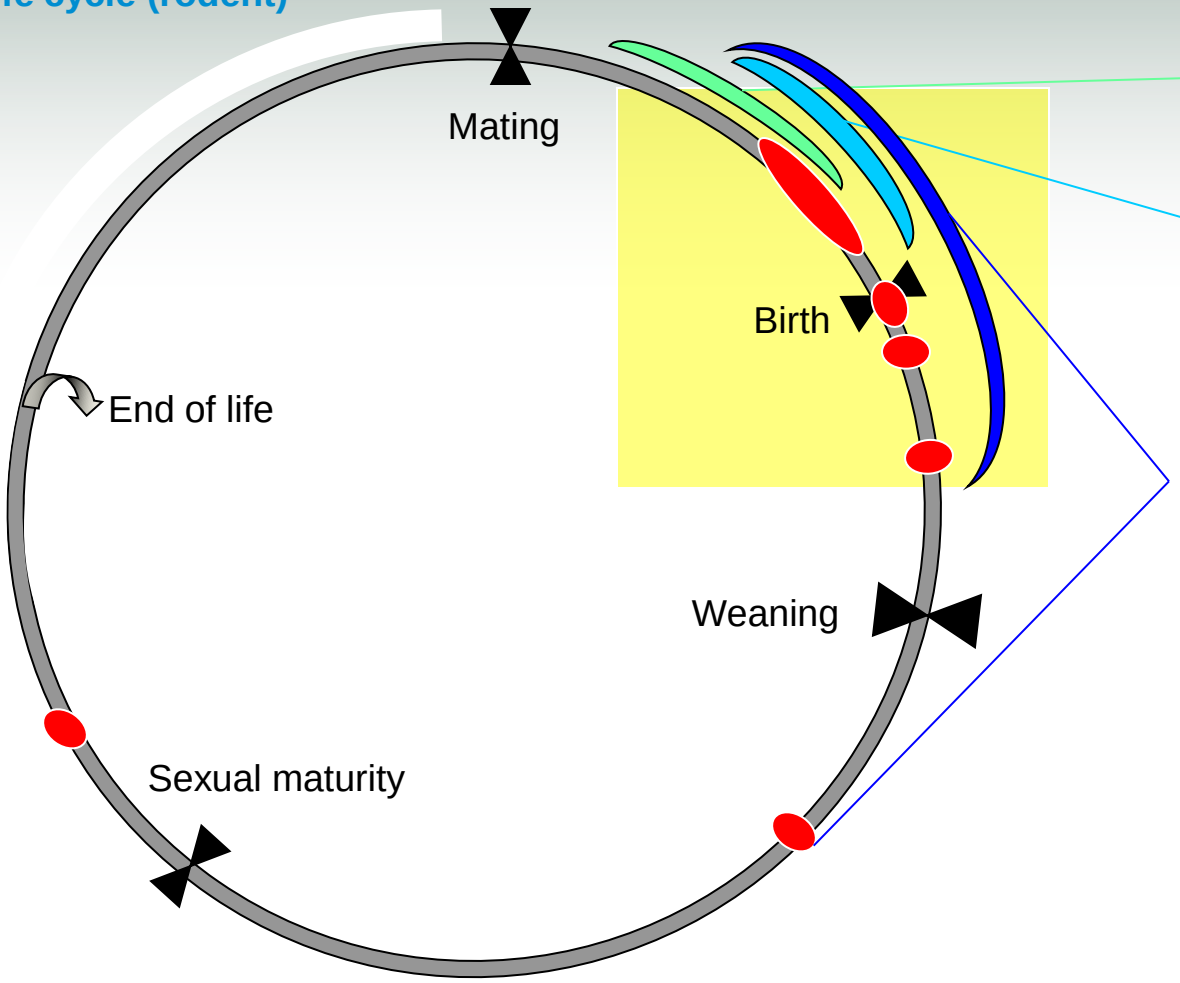


Six Androgen mediated endpoint assessments in males

- *Decreased Testosterone Synthesis*
- *Cryptorchidism and Hypospadias*
- *Ano-genital Distance*
- *Nipple Retention*
- *Sexual Organ Weight Changes*
- *Decreased Sperm Production*

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 Study[1]

DiNP Extended Effects Study[1]

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

= timepoint to assess androgen endpoint

= window of susceptibility

[1]New studies conducted by The Hamner Institutes for Health Sciences

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

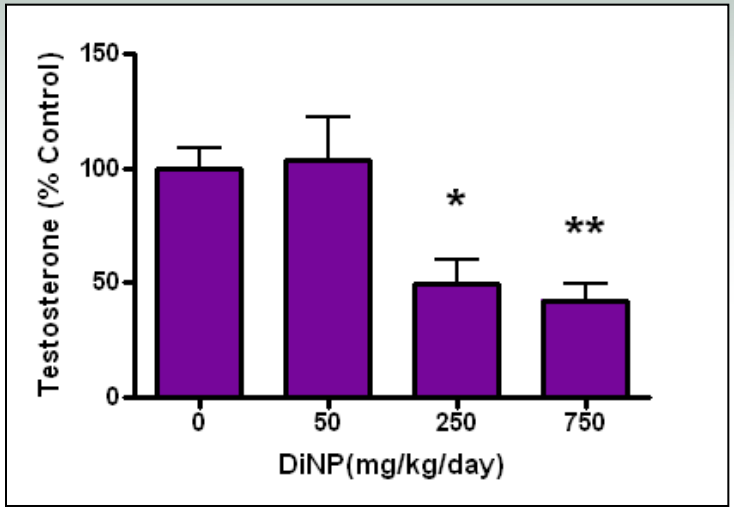
1) Developmental Effects
   GD 19, 20; 2hr, 24 hr post-dose

2) Pharmacokinetics
   GD 19; 0.5 – 24 hr post-dose

3) PBPK Model for maternal and fetal dosimetry

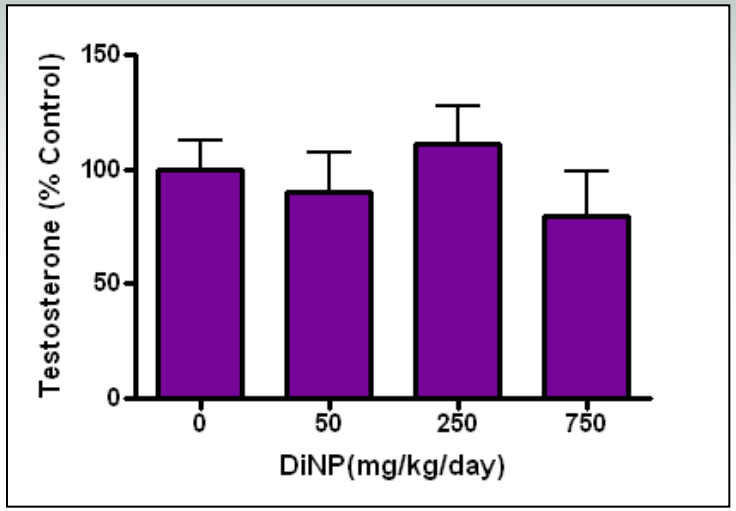
Developmental Effects- Testosterone

2 hr post-dose



* Versus concurrent controls

24 hr post-dose



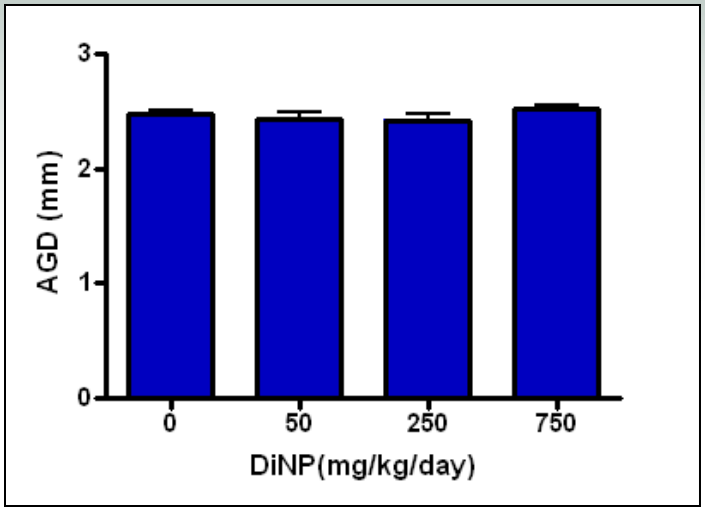
* Versus concurrent controls

- Clear no-observed effect level for DiNP-induced decrease in testosterone at 50 mg/kg/day
- Calculated ED_{50}
  - DiNP = 389 mg/kg/day
  - DBP = 39 mg/kg/day[1]
  - DEHP = 100 mg/kg/day[2]

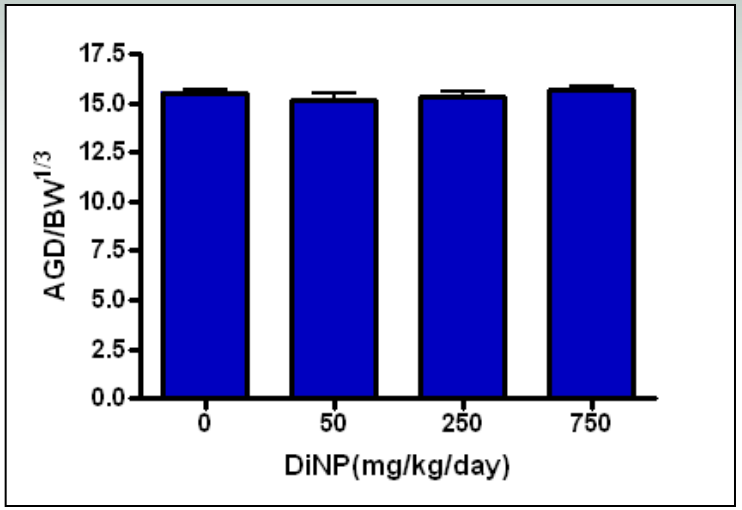
[1]DBP data from Mylchreest et al., 1999 (PND 2)
[2]DEHP data from Borch et al., 2004; Gray et al., 2009 (PND 2)

Developmental Effects- Anogenital Distance (AGD)

Absolute AGD



Scaled AGD



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

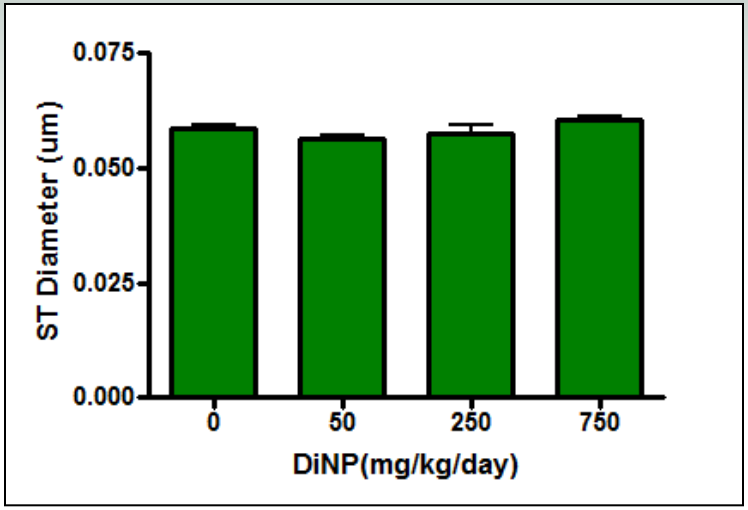
- NOEL
  - DiNP = >750 mg/kg/day
  - DBP = 100 mg/kg/day[1]
  - DEHP = 100 mg/kg/day[2]

- LOEL
  - DiNP = no LOEL identified
  - DBP = 250 mg/kg/day[1]
  - DEHP = 300 mg/kg/day[2]

[1]DBP data from Mylchreest et al., 1999 (PND 2)
[2]DEHP data from Gray et al., 2009 (PND 2)

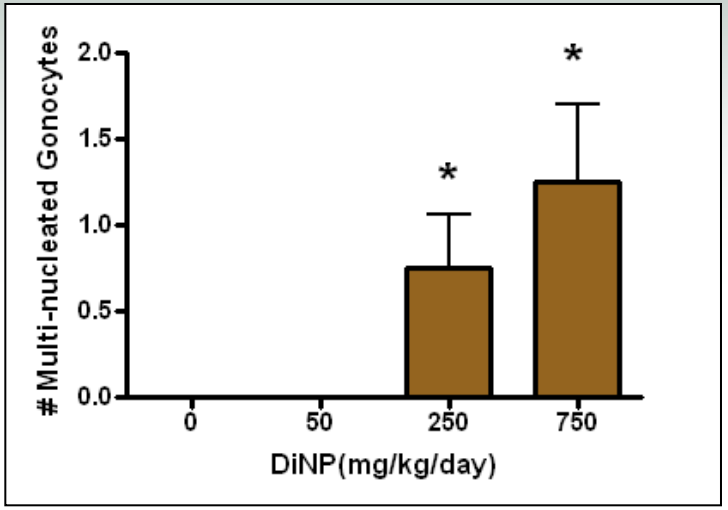
Developmental Effects- Testis Histopathology

Seminiferous Tubule (ST) Diameter[a]



- 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

Multinucleated Gonocytes[b]



- 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

[a]Evaluated quantitatively using ImagePro Software at The Hamner Institutes
[b]Evaluated by Gabrielle Willson, Experimental Pathology Laboratories, Inc.

Developmental Effects- Testis Histopathology*

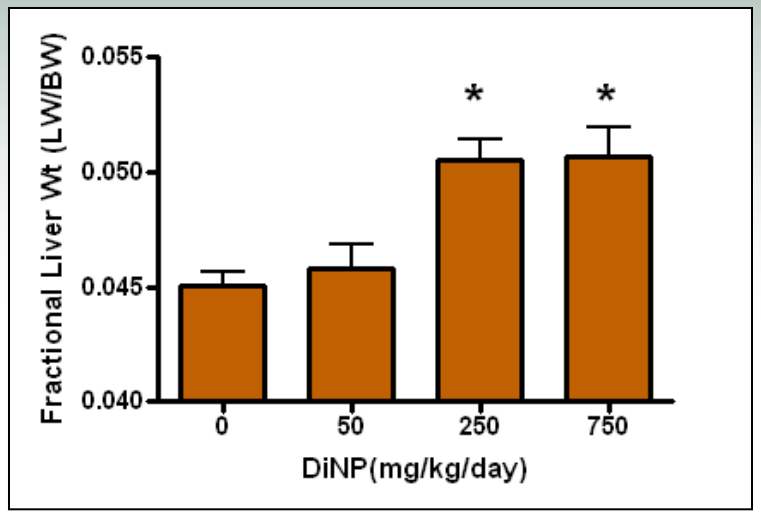
DiNP Dose (mg/kg/day)	Control	50	250	750
Number of animals examined	27	8	8	8
# Animals with MNGs	0	0	2	6*
# Animals with large Leydig cell aggregates	2	3	1	7*
# Animals with increased number of gonocytes	0	0	0	2

- 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

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

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 proliferation[1]

- NOEL
  - DiNP = 50 mg/kg/day
  - DBP = 200 mg/kg/day[2]
  - DEHP = 50 mg/kg/day[2]

- LOEL
  - DiNP = 250 mg/kg/day
  - DBP = 1000 mg/kg/day[1]
  - DEHP = 200 mg/kg/day[1]

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

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

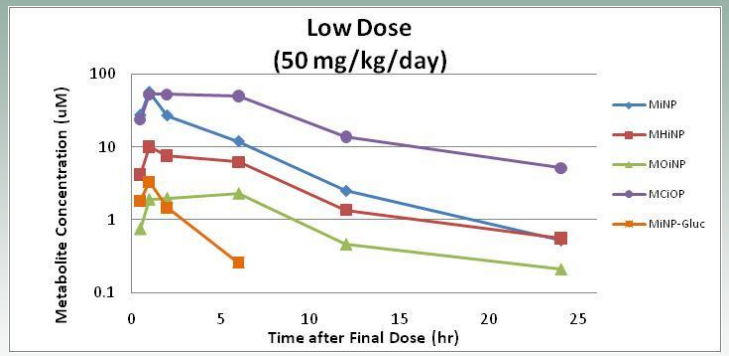
Gestation Study – DiNP Pharmacokinetics

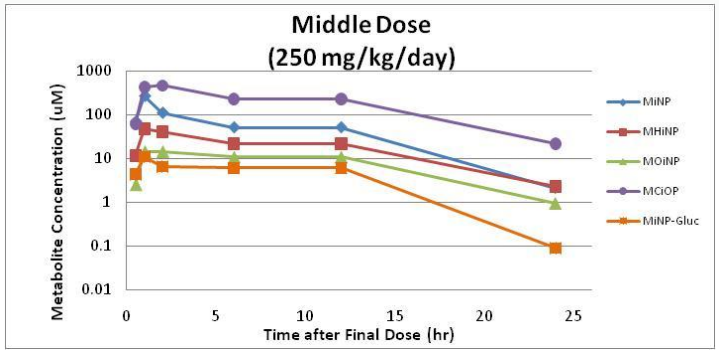
- Preparation of MiNP-glucuronide standard
  - Glucuronide conjugation performed using human liver microsomes
  - Isolation of glucuronide by liquid chromatography
  - Confirmation of MiNP-G by NMR spectroscopy
  - Measure MiNP-G concentration by HPLC-UV/Vis

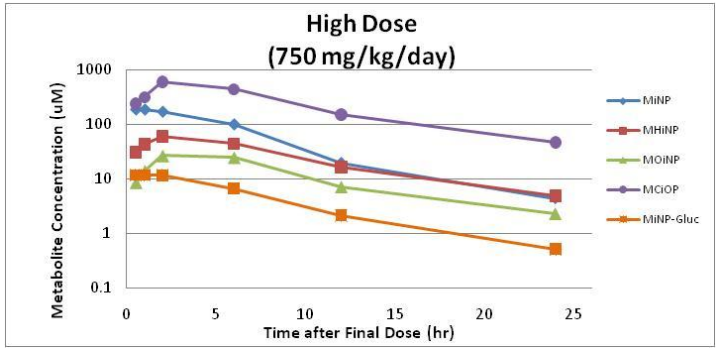
- Metabolite Analysis
  - LC-MS/MS
    - Atmospheric pressure ionization
    - Selective ion monitoring/product ion identification
    - Quantitation by isotope dilution, using ^{13}C-internal standards

- Tissues evaluated
  - Maternal plasma
  - Pup plasma
  - Urine
  - *Maternal liver, placenta*
  - *Fetal testes, amniotic fluid*

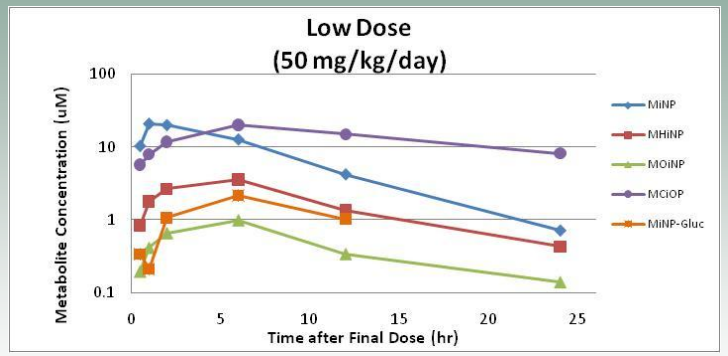
Maternal Plasma

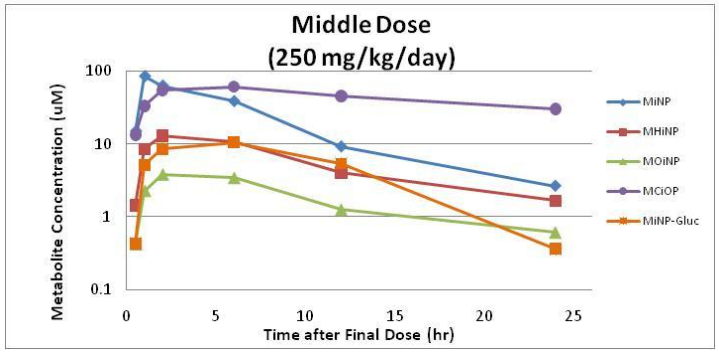


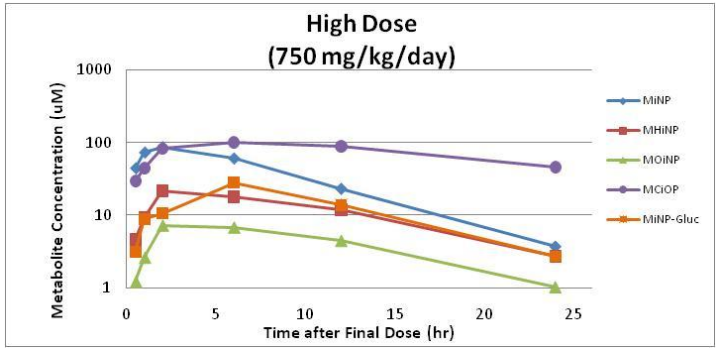




Fetal Plasma





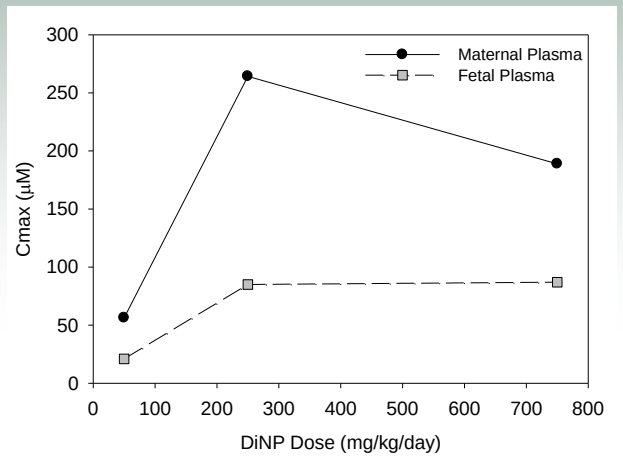


- Concentration of metabolites in maternal plasma: MCiOP> MiNP> MHiNP> MOiNP> MiNP-G
- MiNP-G detected at all doses indicating that glucuronide-conjugation occurs in the rat

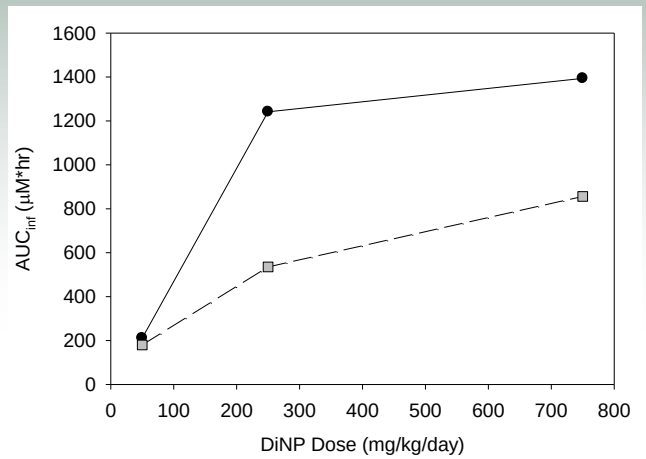
- Concentration of metabolites in fetal plasma: MCiOP> MiNP> MHiNP, MOiNP,and MiNP-G
- MiNP-G generally higher than the maternal plasma

Plasma PK Analysis - MiNP

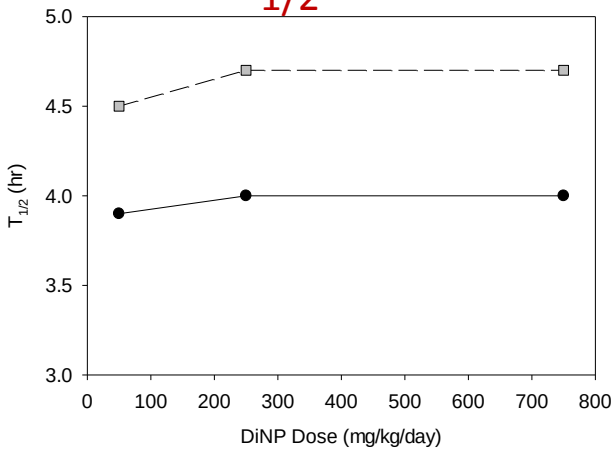
Cmax



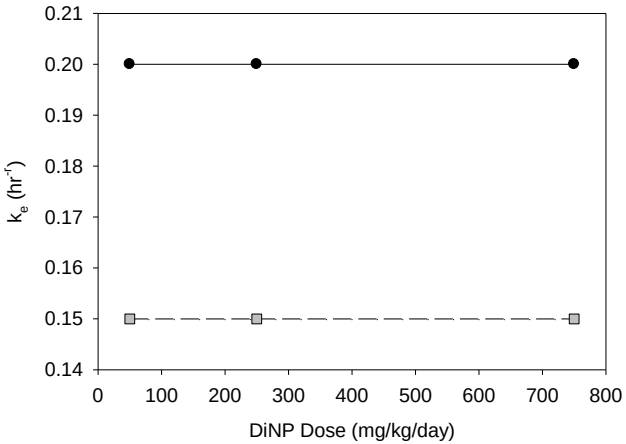
AUC



$T_{1/2}$

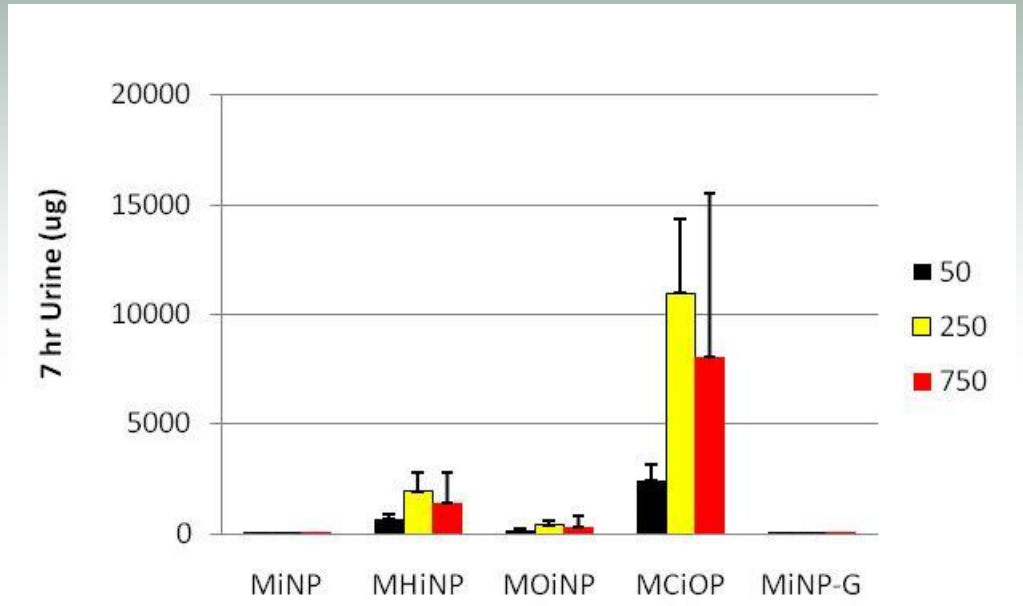


Ke



- 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.

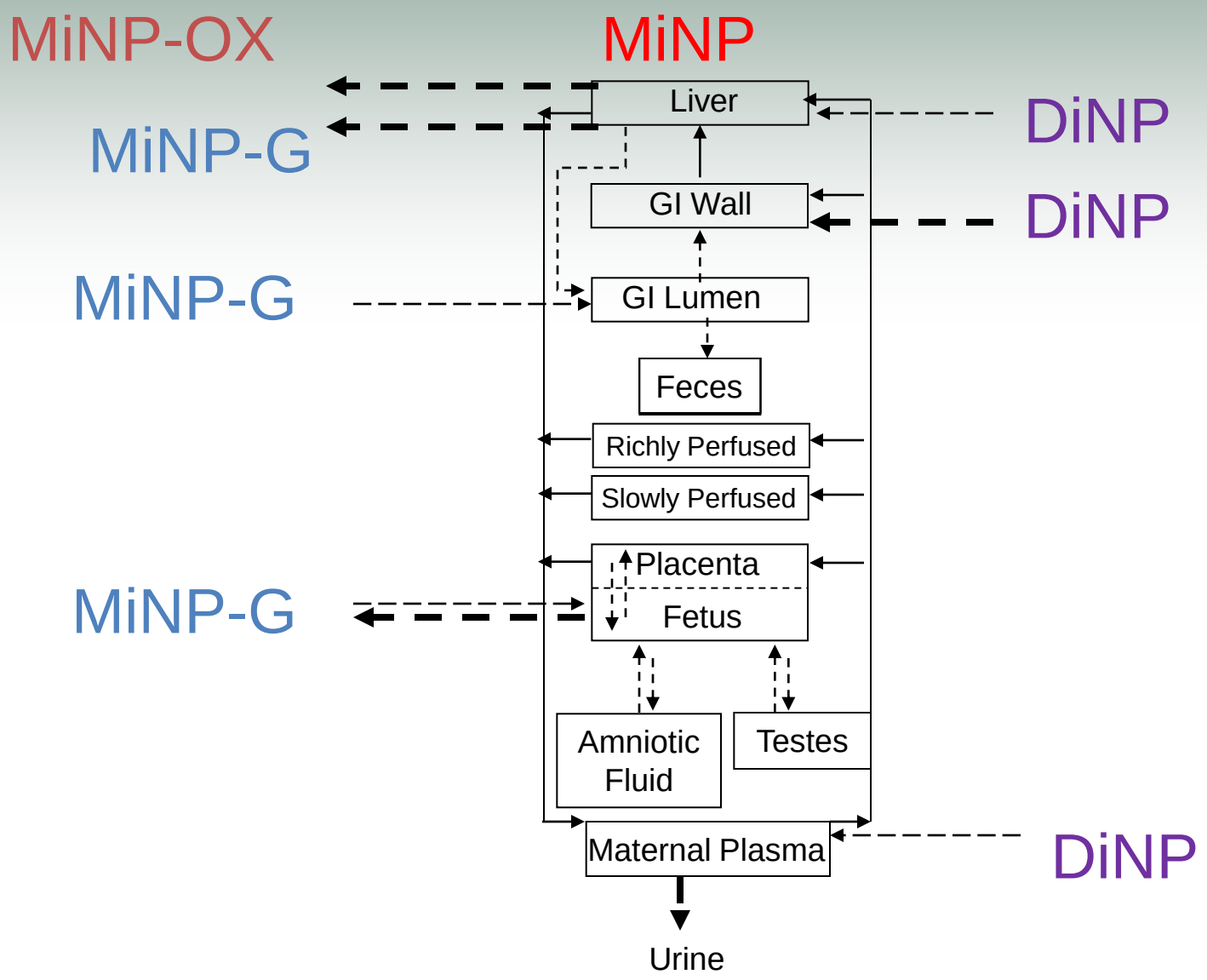
PBPK Model for DiNP – Preliminary Analysis

- DEHP Model Structure
  - Glucuronidation turned off
  - Combined oxidative metabolites (MiNP-OX = MHiNP + MOiNP + 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

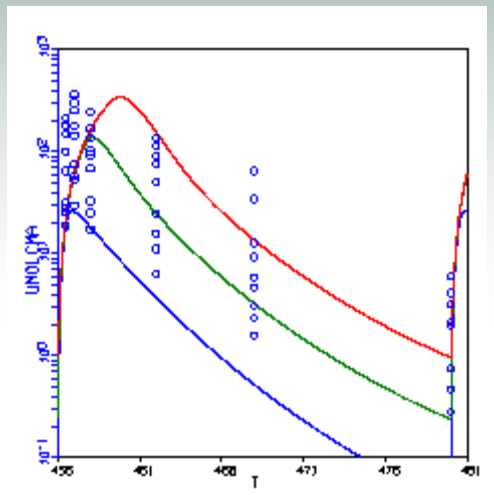


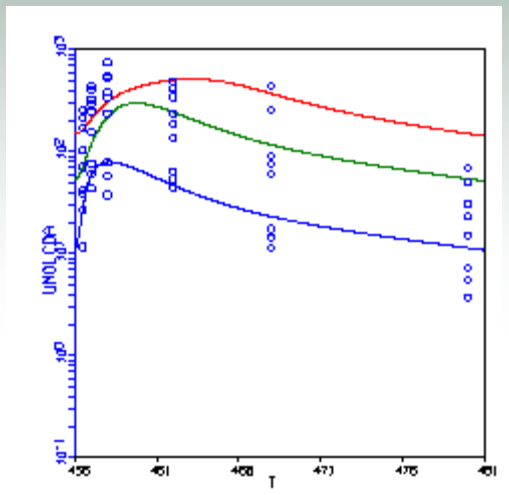
DiNP PBPK Model – Initial fits

MiNP

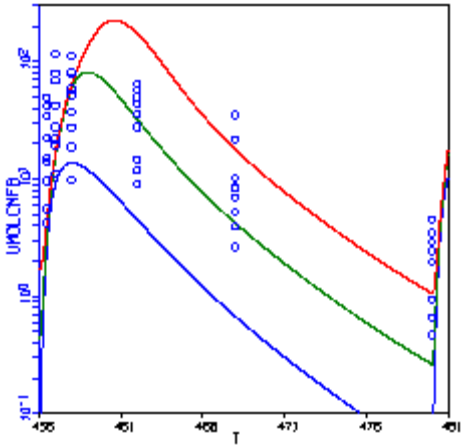
MiNP-Ox

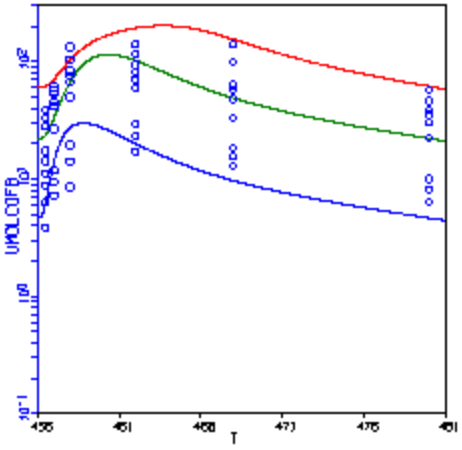
Maternal Plasma





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).

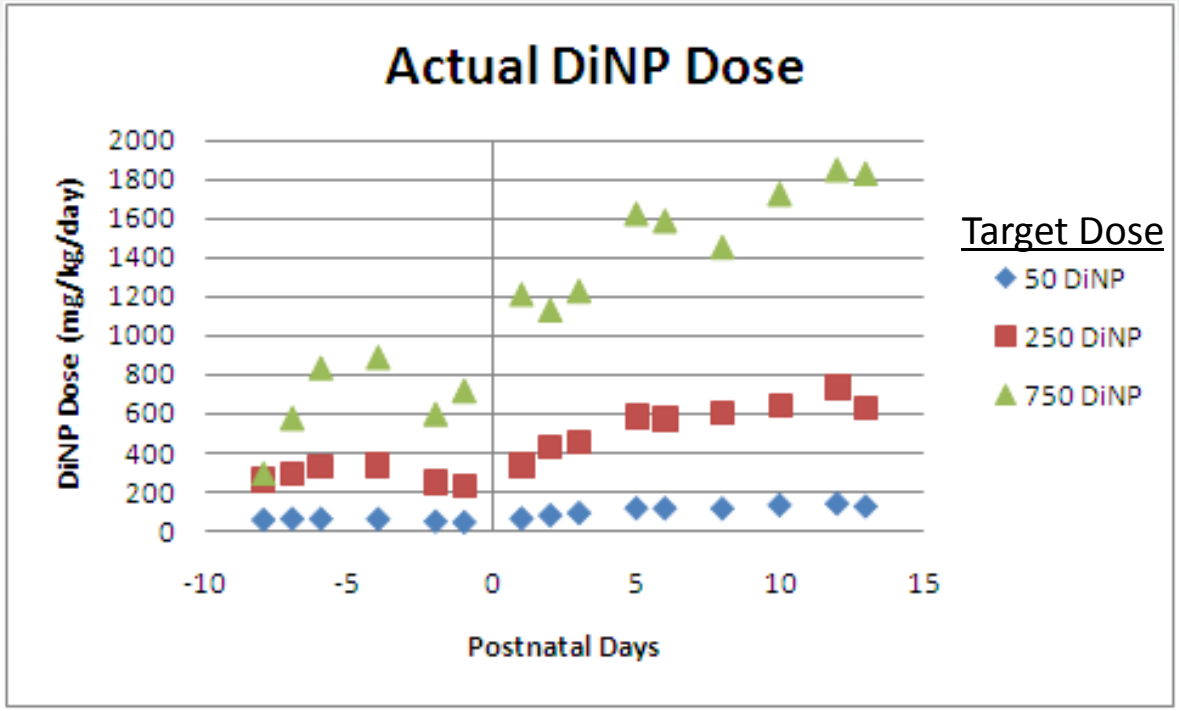
- Study Design
  - Very large n for statistical significance: 20 – 24 litters per treatment group
  - Begin dietary dosing GD 12
  - Weigh dams and food 4x per week
  - All necropsies and observations completely BLINDED
  - PND 2: necropsy 1 male, cull to 8 pups/litter
  - PND 21: euthanize all females
  - 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

[A]Note: Results in this presentation are revised from previous presentationfollowing completed QA/QC review by The Hamner Quality Assurance Unit. Finalized results will be provided in the signed final report.

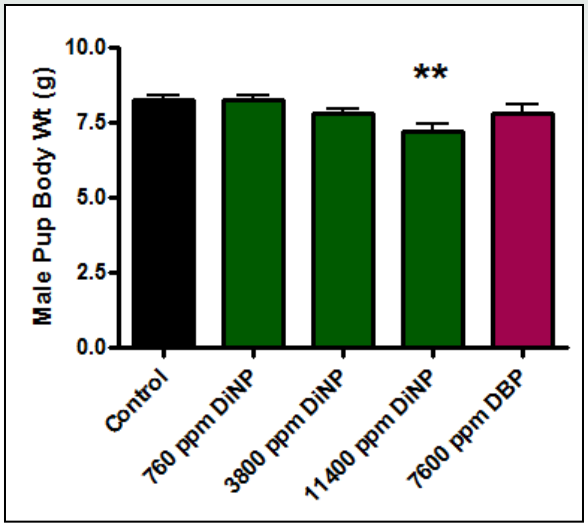
DiNP Dose

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



Postnatal Effects Study – PND 2

Body Weight (males)

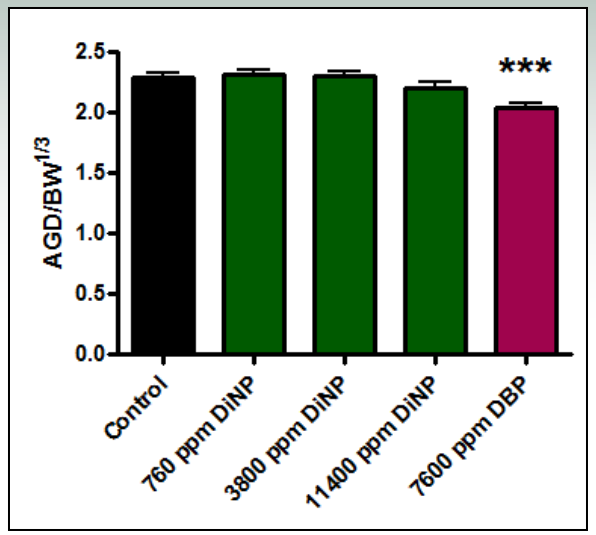


- Pup body weights were reduced in the 11400 ppm DiNP group on PND 2

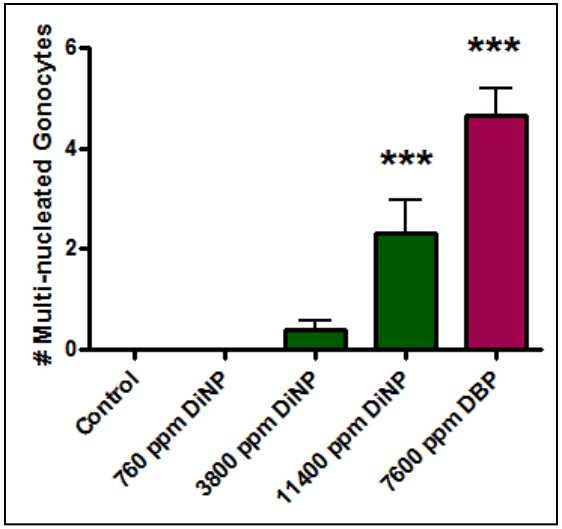
Postnatal Effects Study – PND 2

- 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*

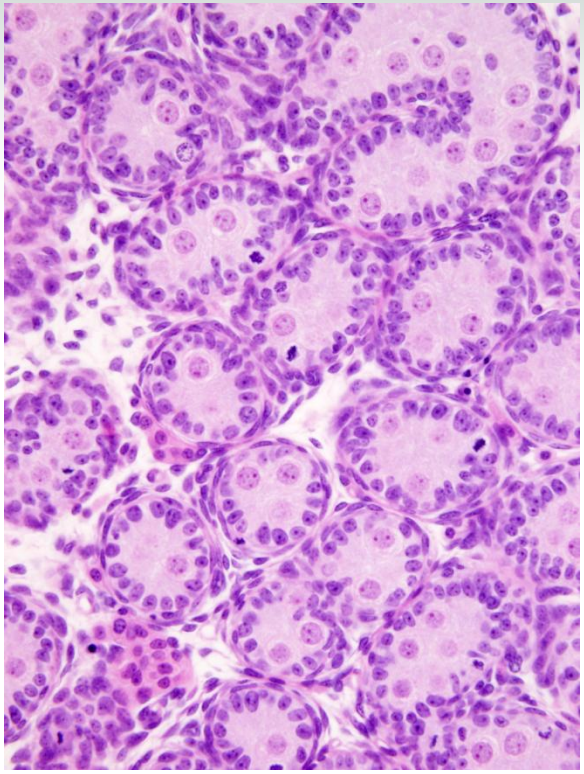
	Control	DiNP 760 ppm	DiNP 3800 ppm	DiNP 11400 ppm	DBP 7600 ppm
Number of animals examined	24	20	20	19	21
# Animals with MNGs	1	2	7*	18**	21**
# Animals with large Leydig cell aggregates	4	4	8	19**	18**
# Animals with increased number of gonocytes	0	0	0	0	5*

- 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

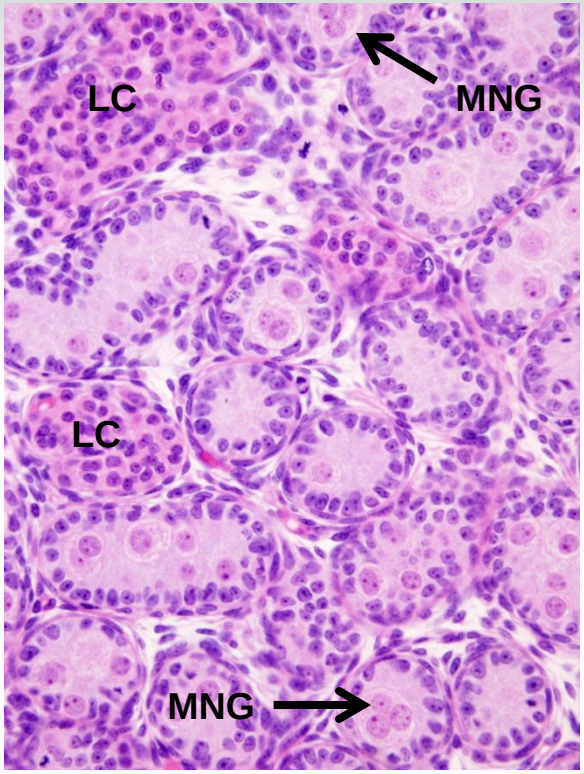
- Statistically significant increase in the number of animals with large leydig cell aggregates at 750 mg/kg/day DiNP

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

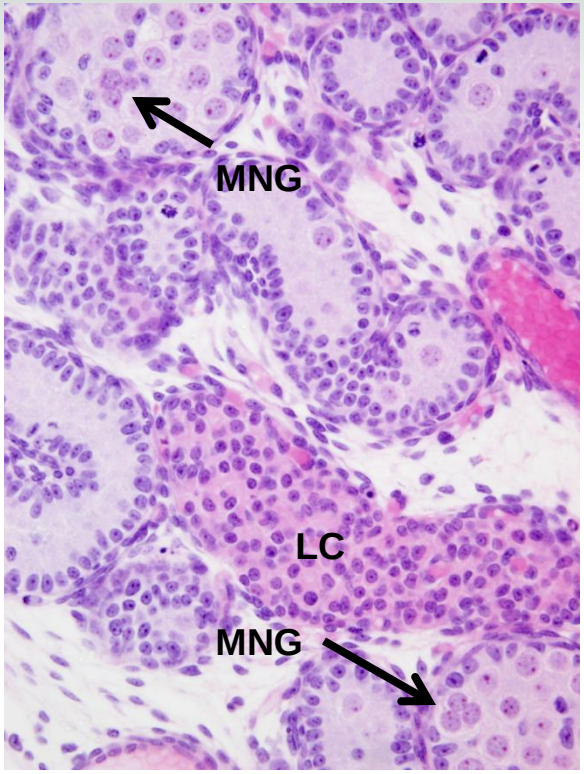
Postnatal Effects Study – Histopathology



Control



11400 ppm DiNP

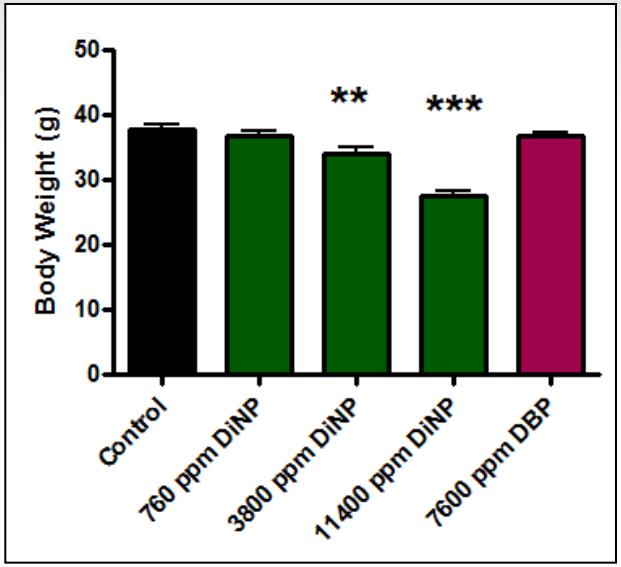


7600 ppm DBP

MNG = Multinucleated gonocytes
LC = Large Leydig cell aggregates

Postnatal Effects Study – PND 14

Body Weight (males)

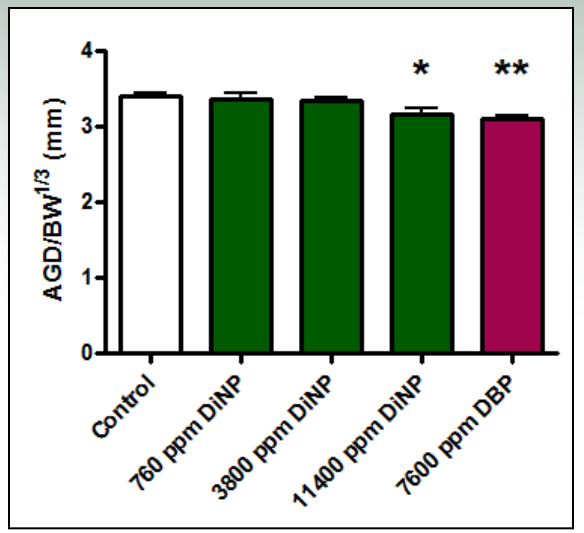


- Pup body weights were reduced in the 3800 and 11400 ppm DiNP groups on PND 14

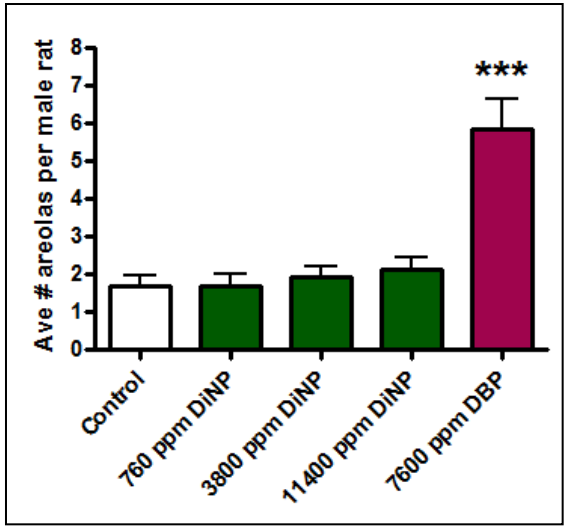
Postnatal Effects Study – PND 14[A]

- 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

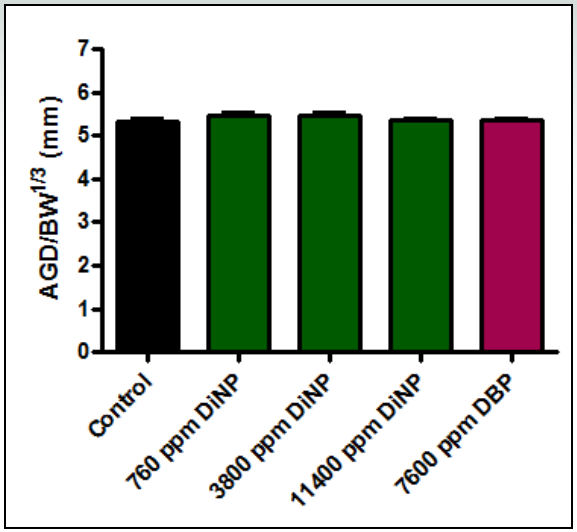


[A]Revised from July 2011 slides

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*

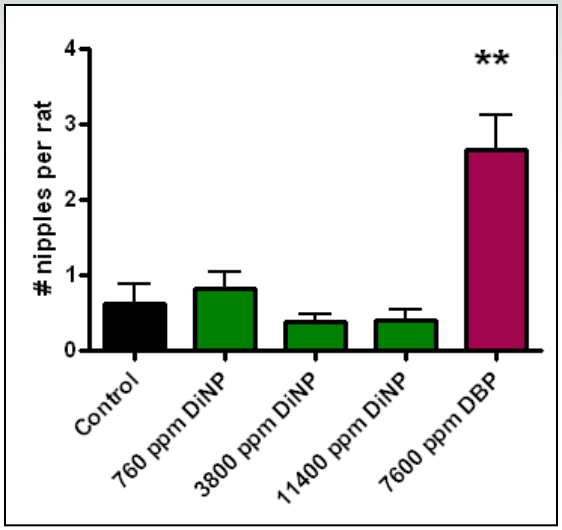
Postnatal Effects Study – PND 49

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



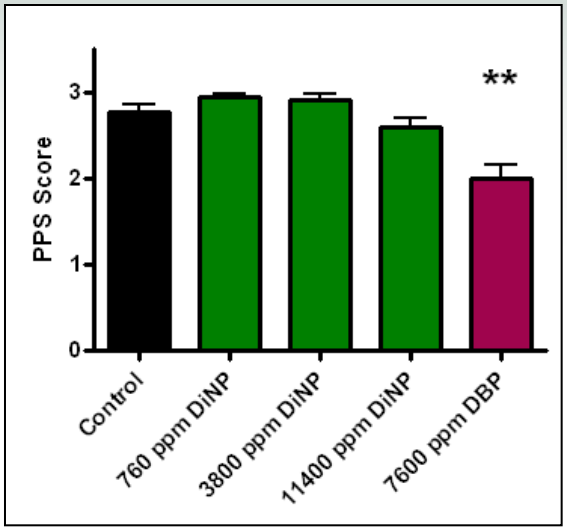
- No effect on absolute or scaled AGD for either DiNP or DBP

Nipple Retention



- No increase in nipple retention with DiNP
- clear increase observed with DBP

Preputial Separation



- 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

[a]Includes 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

[A]Table 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	• ≥ 250 mg/kg/day DiNP
– No body weight effects	– PND 2 body weight (750 mg/kg) – PND 14 body weight (≥250 mg/kg)
– Nipple retention – AGD (absolute and scaled) PND 2 + 14 – Phallus development – Epididymal development – Preputial separation – Weight of 4 reproductive organs	– PND 14 reduced AGD (750 mg/kg)
– PND 2 ST – some enlarged tubules – PND 2 #MNG/section, large LC aggregates • Effects were seen to be transient (not observed at PND 49)	– 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)

[A]*Table revised from July 2011 slides*

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