

# **CLARITY-BPA Core Study: A Perinatal and Chronic Extended-Dose-Range Study of Bisphenol A in Rats**

## **Overview of Core Study Design**

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- Study aim
- Overview of study design
- Discussion of BPA background exposure



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- To characterize the toxicological potential of bisphenol A (BPA) following a perinatal only or a chronic exposure in male and female rats exposed to a wide range of BPA levels



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- NCTR Sprague-Dawley rat (strain SD/CD23/NctrBR)
  - Previously used at NCTR in the NTP's "Endocrine Disruptor Initiative" and NCTR 90-day BPA subchronic studies
  - Comprehensive characterization of BPA pharmacokinetics across life stages and tissues





# Rationale for Special Animal Housing

- Sources of BPA exposure in animal rooms:
  - Polycarbonate plastic material (such as cages and water bottles)
  - Feed
    - Camacho *et al.*, 2016: all four natural ingredient diets and two purified ingredient diets assayed had detectable levels of BPA (range: 0.46-3.64 ppb)
- Sources of estrogenic exposure in animal rooms:
  - Feed (phytoestrogens, if using soy- and alfalfa-based feed)
  - Bedding (mycoestrogens, if using contaminated corn husks bedding)



- Polysulfone cages with wire lids and polysulfone microisolator tops
- Glass water bottles with metal sip tubes
- Millipore-filtered water provided *ad libitum*
- Hardwood chip bedding
  - AlphaDri cellulose bedding, when recommended by Veterinary Services (e.g., pododermatitis, ventral masses)
- All housing materials certified to contain BPA levels below the analytical background





- 5K96 Purina irradiated pellets provided *ad libitum*
  - Low phytoestrogen, casein-based, soy- and alfalfa-free chow
  - Previously used at NCTR in combination with this animal model in the NTP's "Endocrine Disruptor Initiative" and NCTR 90-day BPA subchronic studies
- All feed lots analyzed prior to use for:
  - Dietary components (%protein, %fat, vitamins, etc.)
  - Contaminants (heavy metals, pesticides, aflatoxins, etc.)
  - Phytoestrogens (genistein, daidzein, and coumestrol)
  - Mycoestrogen (zearalenone)
  - BPA

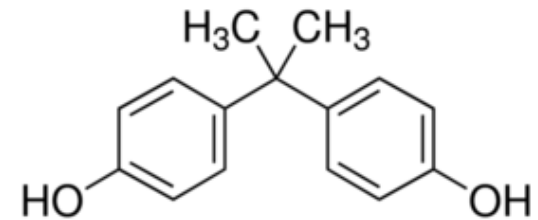


# Xenoestrogens and BPA in Feed

- Eleven feed lots used in the study contained an average of:
  - 1.79 ppm genistein
  - 1.66 ppm daidzein
  - 0.01 ppm coumestrol
  - 0.005 ppm zearalenone
  - 1.28 ppb BPA
    - ~0.03-0.2  $\mu\text{g}$  BPA/kg body weight (bw)/day
    - ~1-8% of the lowest BPA dose in the study



- Bisphenol A (BPA, CAS # 80-05-7)

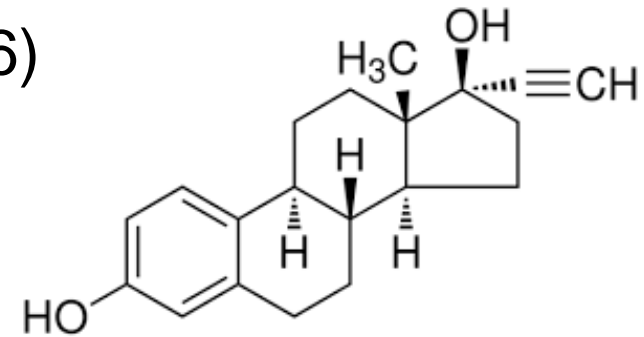


- BPA solid was air-milled to ensure homogeneity of dosing suspensions
- Comprehensive chemical analysis conducted by Battelle (Columbus, OH)
- Identity and purity (>99%) re-certified at NCTR by proton nuclear magnetic resonance (NMR) and high performance liquid chromatography with photodiode array detection (HPLC-PDA) at start, middle, and end of study



# Ethinyl Estradiol Test Article

- Ethinyl estradiol (EE<sub>2</sub>, CAS # 57-63-6)
  - Reference estrogen control



- Identity and purity (>99%) certified at NCTR by NMR and HPLC-PDA at start, middle, and end of study



- Vehicle control group: 0.3% aqueous carboxymethylcellulose
  - To ensure homogeneity of dosing suspensions
- Five BPA groups: 2.5, 25, 250, 2,500, and 25,000  $\mu\text{g}/\text{kg}$  bw/day
  - Lowest dose set to be  $\geq 10$ -fold higher than allowed dietary intake of BPA
  - Highest dose set to afford sufficient margin of exposure to current estimated human exposure to BPA
- Two EE<sub>2</sub> groups: 0.05 and 0.5  $\mu\text{g}/\text{kg}$  bw/day
  - 10-fold lower than doses used in NCTR 90-day subchronic BPA study



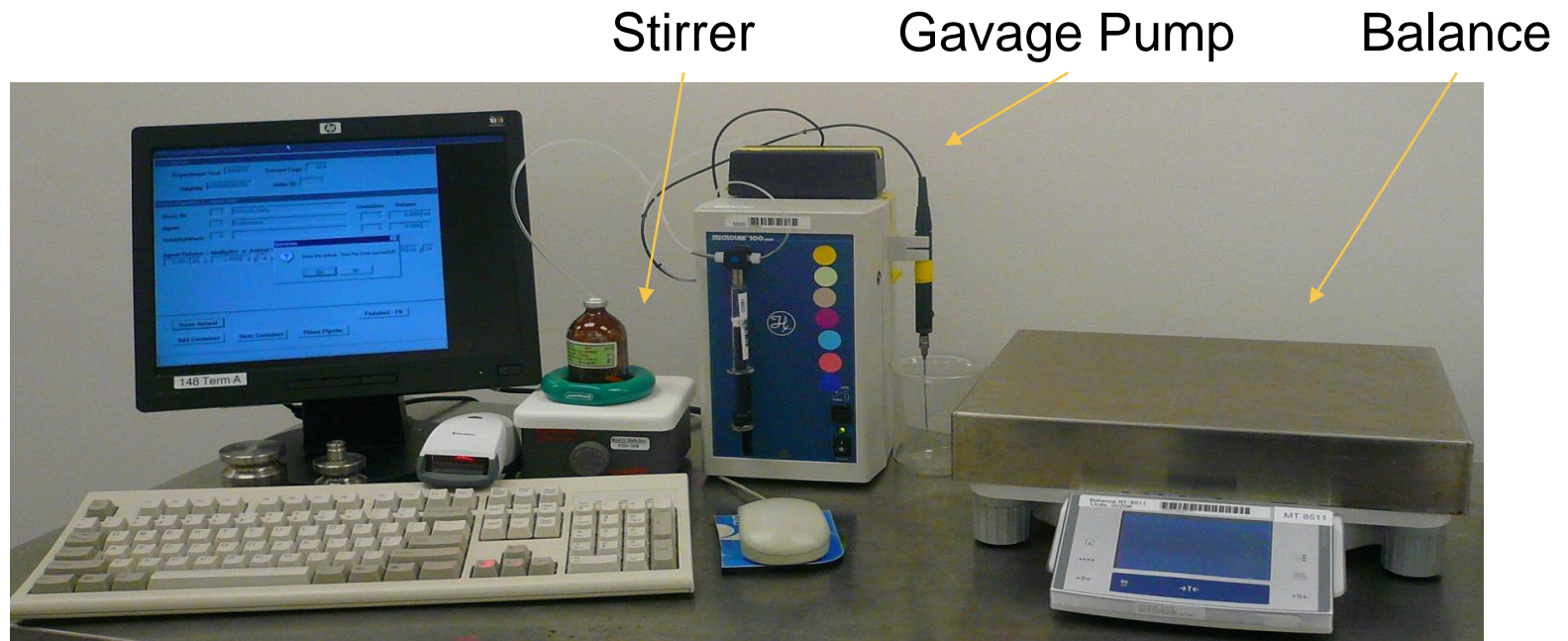
- Certified to be within  $\pm 10\%$  of the target concentrations
- Used within the certified window of stability
- Certified to be homogeneous ( $\pm 10\%$ )
- Verified periodically over the course of the study
  - To confirm that label in dosing vial matched dosing formulation



- Dosing route: daily oral exposure by gavage, 7 days a week
- 5 mL/kg bw/day
  - Based on daily (prior to postnatal day 90) or weekly (after postnatal day 90) body weight
- Automatically dispensed by a Hamilton Microlab 500 pump
  - Certified to deliver within  $\pm 10\%$  of target dose
  - Four dosing stations per animal room
  - Doses administered from low to high within each station
  - Pumps thoroughly flushed between dosing formulations



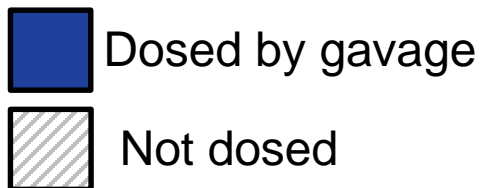
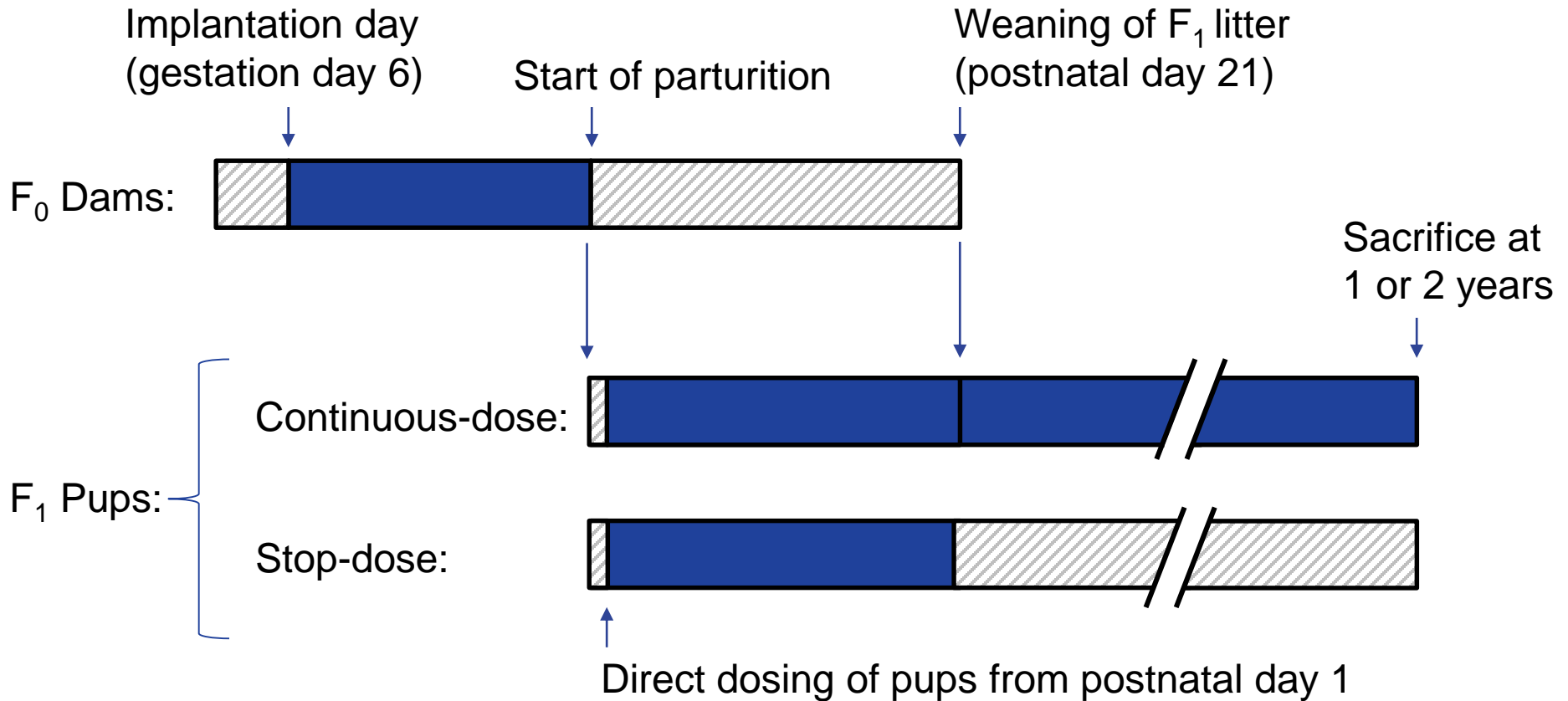
- Pump 1: Vehicle
- Pump 2: BPA (2.5, 25, and 250  $\mu\text{g}/\text{kg}$  bw/day)
- Pump 3: BPA (2,500 and 25,000  $\mu\text{g}/\text{kg}$  bw/day)
- Pump 4: EE<sub>2</sub> (0.05 and 0.5  $\mu\text{g}/\text{kg}$  bw/day)







# Dosing Regimen





# Rationale for Dosing Regimen

- Continuous-dose arm: standard guideline exposure for chronic toxicity studies that include perinatal exposure
- Stop-dose arm: perinatal-only exposure
  - Included due to interest in assessing potential effects of hormonally-active compounds upon perinatal-only exposure
  - Animals not dosed after weaning at postnatal day 21
  - Stop-dose arm did not include EE<sub>2</sub> dose groups due to logistical limitations
- Direct dosing of F<sub>1</sub> pups after birth, to overcome the poor lactational transfer of BPA



- Five matings (referred to as “loads”), spaced 4 weeks apart
- 120 breeding pairs per load
  - Number of breeding pairs selected based on past reproductive performance (mating and littering success, including litter size)
- No mating between litter mates or “first-cousins” allowed
- Breeders placed under study conditions at weaning (postnatal day 21)
- Allocation period: Sept 1, 2012 through Dec 22, 2012



- Breeders paired and allowed to mate for up to 10 days
- Daily check for vaginal plug or collection of vaginal smear
- Gestation day 0 = day when a vaginal plug or a sperm-positive vaginal smear was noted
- Breeders removed from the study if no evidence of mating after 10 days of mating



- Dams were removed from the study:
    - If they produced a litter prior to gestation day 20
      - Indicative that day of mating had been missed
    - If they did not deliver a litter by gestation day 26
    - If they produced a litter with less than 3 males or 3 females
    - When their pups were weaned at postnatal day 21
- Assessed for uterine implantation sites
- No differences in the number of uterine implantation sites among dose groups



- F<sub>1</sub> pups were not allocated to the study if they originated from litters with insufficient size (less than 3 pups per sex)
- Litters were randomly culled to a maximum of 5 males and 5 females on postnatal day 1
- Litters were weaned at postnatal day 21
- Up to 3 males and 3 females per litter were allocated to the chronic study
  - No same-sex litter mates were assigned to the same dose group within dosing arm and sacrifice time



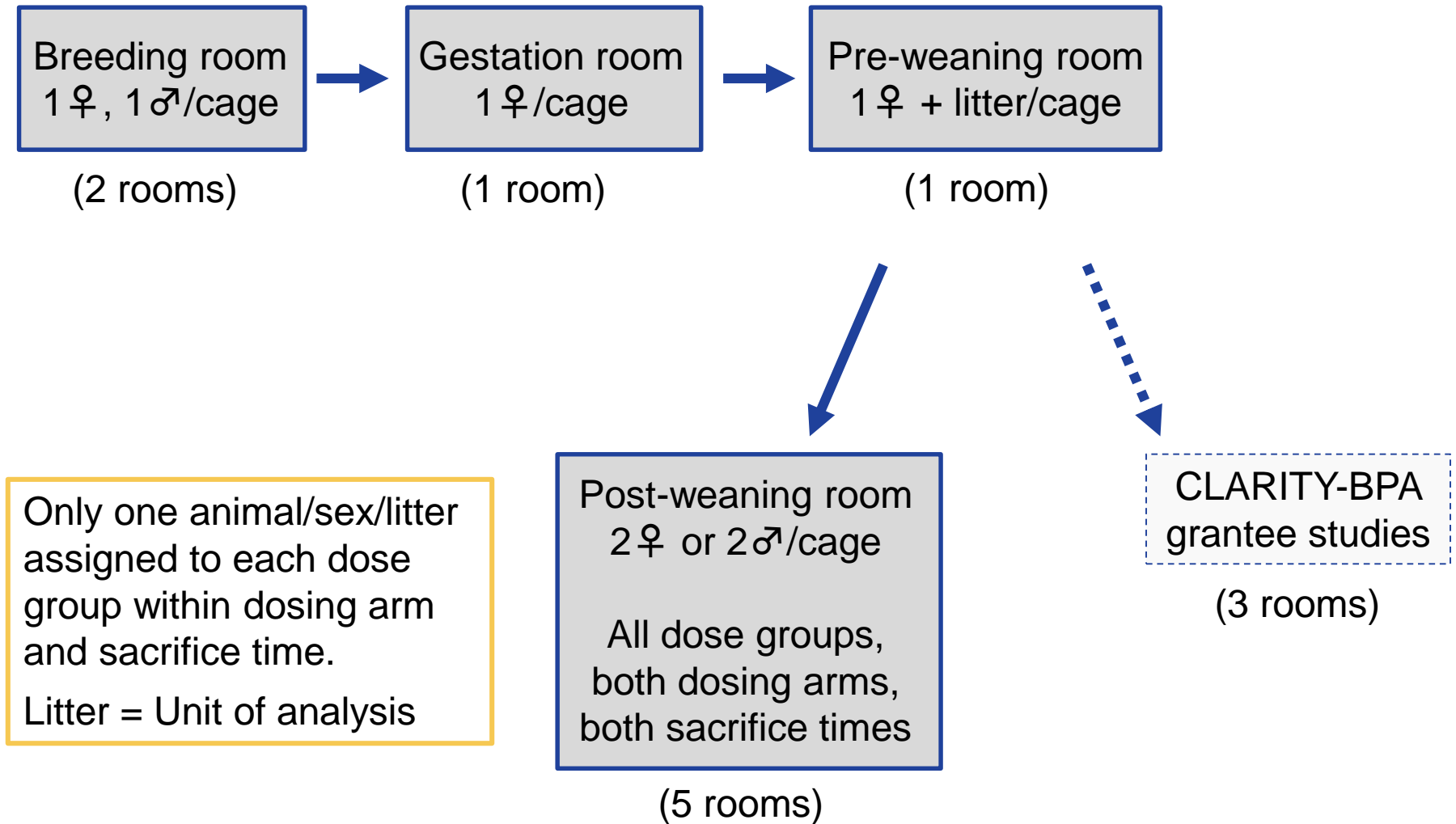
# F<sub>1</sub> Animal Allocation at Weaning

Sacrifice Time	Dosing Arm	Dose Group	Animals/Sex/Dose Group
1-year* (interim)	Continuous	Vehicle, BPA, EE <sub>2</sub>	20-26
	Stop	Vehicle, BPA	19-22
2-years (terminal)	Continuous	Vehicle, BPA	46-50
		EE <sub>2</sub>	26
	Stop	Vehicle, BPA	46-50

\*Original target for 1-year groups was 26/sex/dose group, but this was reduced to afford animals to CLARITY-BPA grantee studies



# Housing of Animals







# In-Life Endpoints

Endpoint	Frequency
Morbidity/mortality	Twice daily
Clinical observations	Weekly or as noted
Individual F <sub>0</sub> dam body weight	Gestation day 6 through start of parturition
Number of F <sub>1</sub> pups alive/dead	Postnatal days 0 (= day of birth) and 1
F <sub>1</sub> litter sex ratio	Postnatal day 1
F <sub>1</sub> litter weight per sex	Postnatal day 1
Individual F <sub>1</sub> pup body weight	Postnatal day 1 (after litter culling) through 21 [ <i>stop-dose arm</i> ] or 90 [ <i>continuous-dose arm</i> ]; weekly afterwards
F <sub>1</sub> vaginal opening and cytology	( <i>next slide</i> )
F <sub>1</sub> palpation	Weekly, starting at 6 months of age
F <sub>1</sub> feed consumption*	Weekly until ~postnatal day 90, monthly afterwards

\*Data collected to estimate the background dietary intake of BPA



# Vaginal Cytology Endpoints

- Randomly selected 26 females per dose group per dosing arm assigned to the terminal sacrifice
- Monitoring for vaginal opening from postnatal day 22
- Collection of vaginal smears for 14 consecutive days at 16 weeks of age to assess estrous cycle length (per stage and in total)
- Collection of monthly vaginal smears for 5 consecutive days to assess estrous cyclicity and reproductive senescence
  - Animals showing 3 consecutive days of estrus (including estrus, estrus/diestrus, and proestrus/estrus) or 5 consecutive days without a day of estrus for 2 consecutive months were considered to have started aberrant cycles and were no longer monitored



# Interim (1-Year) Sacrifice Endpoints

- Hematology
- Clinical chemistry
- Sperm parameters: testicular spermatid head counts and cauda sperm counts, motility, and morphology
- Body and organ weights
- Histopathology
  - Adrenals, aorta (thoracic), bone marrow (femur), brain, right epididymis, heart, kidneys, liver, 5th left mammary gland (inguinal), ovaries, oviduct, pancreas, pituitary, prostate (dorsolateral and ventral), seminal vesicles with coagulating gland, spleen, right testis, thymus, thyroid, uterus, vagina, gross lesions
  - Six step sections cut at 100  $\mu\text{m}$  intervals evaluated for **dorsolateral prostate (text revised 04/27/2018)**



# Terminal (2-Year) Sacrifice Endpoints

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- Body weight
- Histopathology
  - As described for interim sacrifice



# Histopathological Evaluation

- Performed by the Study Pathologist
- Evaluated by an independent quality assessment (QA) group
  - Pathology data review conducted by the QA pathologists
  - 100% review of slides from ovary, uterus, vagina, testes, epididymis, prostate, seminal vesicle, pituitary (♀, ♂), and mammary gland (♀, ♂)
- Reviewed by a Pathology Working Group who evaluated:
  - Potentially treatment-related lesions
  - Disagreements in diagnosis between the Study Pathologist and the QA pathologists
  - Rare spontaneous lesions



- Data analyzed within sex, dosing arm, sacrifice time, and test article
- Litter was the unit of analysis
- NTP standard statistical procedures
  - Exception: additional tests for histopathology data (*next slide*)
- Tests applied to non-histopathology data were two-sided and corrected for multiple comparisons
  - Exception: Trends for abnormal estrous cycle were one-sided
- Histopathology data analysis was one-sided and not corrected for multiple comparisons
- Statistical significance at 0.05 level



# Statistical Analysis of Histopathology Data

- Primary statistical tests: lesion incidence only  
(no assumption of monotonicity across dose levels)
  - Interim sacrifice: Cochran-Armitage test for trend and Fisher's exact test for pairwise comparisons (CAFE test; no adjustment for survival)
  - Terminal sacrifice: Poly-3 method (survival-adjusted)
- Secondary statistical tests: lesion severity and incidence  
(no adjustment for survival)
  - Jonckheere-Terpstra test for trend and Shirley-Williams test for pairwise comparisons (JT/SW test)
    - Assumes monotonicity across dose levels
  - Relative Treatment Effect (RTE) test
    - Does not assume monotonicity across dose levels



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- Known: via feed
  - Maximum BPA in feed allowed: 5 ppb
  - Actual BPA in feed content: 1.28 ppb (range: 0-3.0 ppb)
- Background dietary exposure to BPA was ~0.03-0.2  $\mu\text{g}/\text{kg}$  bw/day
  - ~1-8% of lowest BPA dose in study
  - Based on measured BPA content in feed and feed consumption data



# Background Exposure to BPA

- Presumed: exposure to low levels of BPA via an unknown source, due to housing of load 1 animals in the same room as load 0 grantee animals being dosed with 250,000  $\mu\text{g}$  BPA/kg bw/day
  - Load 1 animals were co-housed with load 0 grantee animals in the gestation and pre-weaning rooms
- Assumption based on findings from the previous NCTR 90-day subchronic BPA study (Churchwell *et al.*, 2014)
  - Inclusion of serum BPA measurements allowed characterization of internal dosimetry and realization of unintended exposure of study animals to BPA
  - Highest BPA dose in animal room: 300,000  $\mu\text{g}$  BPA/kg bw/day
  - Serum BPA-glucuronide levels in the naïve and vehicle controls were similar to those in the lowest BPA (2.5  $\mu\text{g}/\text{kg}$  bw/day) dose group



# Background Exposure to BPA

- Presumed unintentional exposure to BPA in the CLARITY-BPA study could not be tested because:
  - Blood collection for internal dosimetry assessments had not been planned
  - Serum BPA-glucuronide data from NCTR 90-day subchronic study was generated after the co-housing with load 0 animals had ceased
- However, blood was collected from chronic study animals one week prior to interim sacrifice to assess serum levels of BPA-glucuronide
  - Animals from all loads and all post-weaning animal rooms tested
  - Blood collected within 15-60 min of last dose ( $\sim C_{max}$ ) (continuous-dose arm) or at a similar time of the day (stop-dose arm)



# Background Exposure to BPA

- Summary of BPA internal dosimetry data:

Animal Cohort	Highest BPA Dose in Room ( $\mu\text{g}/\text{kg}$ bw/day)	BPA-Glucuronide Detected in Vehicle Serum?
NCTR 90-day subchronic study	300,000	<b>Yes</b> (~ to 2.5 BPA $\mu\text{g}/\text{kg}$ bw/day group)
Chronic study, load 1 while overlapping with load 0	250,000	Not determined
Chronic study, loads 1-5	25,000 (10-fold lower)	<b>No</b>

- Animals potentially exposed to unintentional BPA above background would be those from load 1 (~20-25% animals per dose group)



- Aimed to determine impact of potential unintended exposure to BPA on the statistical analysis outcomes
- Statistical analyses were conducted as described, but excluding all animals that had been housed for any period of time in rooms concurrently with load 0 grantee animals dosed with 250,000  $\mu\text{g}$  BPA/kg bw/day
  - Effects found significant by the sensitivity analysis, but not by the inclusive analysis, are noted in the report tables
  - Effects found significant by the inclusive analysis, but not by the sensitivity analysis, are not noted in the report tables
- Overall findings of the inclusive and sensitivity analyses were similar, indicating that a potential unintentional exposure of animals to BPA did not impact the outcome of the study



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**THANK YOU**