Welcome to the American College of Toxicology's Webinar Series

Understanding Developmental and Reproductive Toxicity Studies

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Presenter (Bio)

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Dr. York is a formally trained toxicologist with 30 years of research experience. He earned his Ph.D. in Toxicology at the University of Cincinnati and completed a two-year postdoctoral fellowship at Children's Hospital's Institute for Developmental Research in Cincinnati. He was board-certified as a Diplomate of the American Board of Toxicology in 1986 and has served 4 years on its Board of Directors. He is certified as a European Registered Toxicologist (2006) and as a Fellow of the Academy of Toxicological Sciences, as well as a Fellow for Toxicology Excellence for Risk Assessment. He has served as a study director on over 700 safety evaluation studies and published over a 100 manuscripts, review articles, book chapters and abstracts.

Dr. York has been a member of the Society of Toxicology since 1985, and the American College of Toxicology since 1998. He is currently President of the Reproductive and Developmental Toxicology Specialty Section of SOT. He has served as President of the Middle-Atlantic Regional Section (MASOT; 2012), the Midwest Teratology Association (MTA; 1989) and Mid- Atlantic Reproduction and Teratology Association (MARTA; 2004). Dr. York has been a member of the Teratology Society since 1984.

He has served as a reviewer for *Toxicology and Applied Pharmacology* and *International Journal of Toxicology* and as a member of the Editorial Board of *Fundamental and Applied Toxicology*. Dr. York is a peer consultant for assessment of the potential health-effect risks for a number of consulting and legal firms and recently served on a FDA GRAS Panel for a food additive. Currently, he is on an EPA SAB panel for trimethylbenzene and an adjunct professor teaching Human Anatomy & Physiology at a college in Syracuse, NY.

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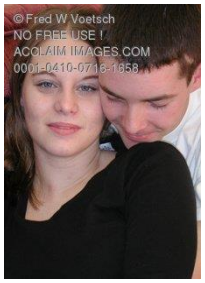
Assessing the Effects of a Toxicant on Reproduction and Development






Risk Assessment

Exposure Assessment	Hazard Characterization	Dose-response Assessment	Risk Characterization



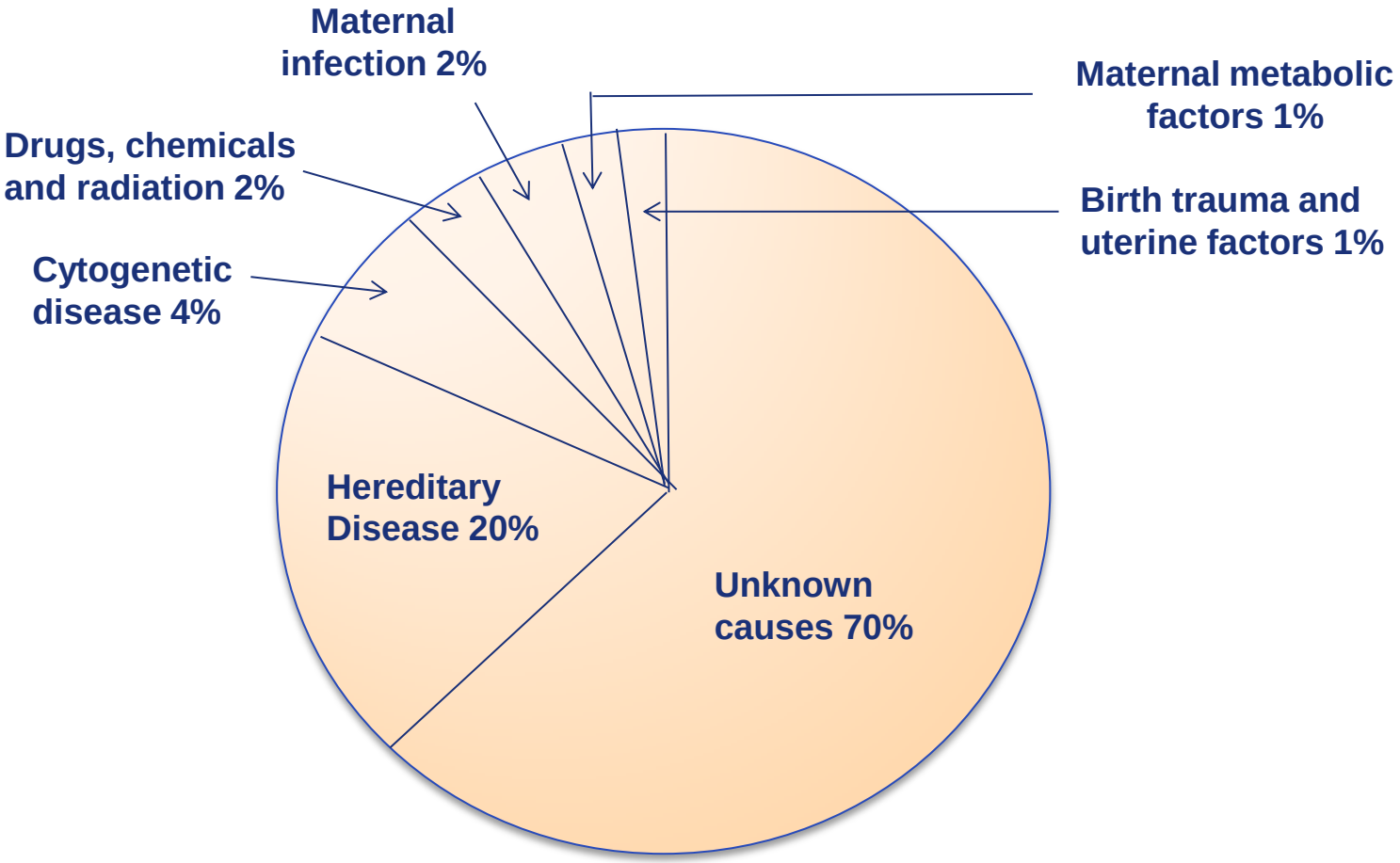


Exposures	Toxicokinetics	Toxicodynamics	Outcome
Oral	Absorption	Receptors	Normal
Dermal	Distribution	Cell Types	Death
Inhalation	Metabolism	Cell-cell interactions	Malformation
Intravenous	Elimination	Specific signaling	Growth retardation
		Differentiation	Altered function

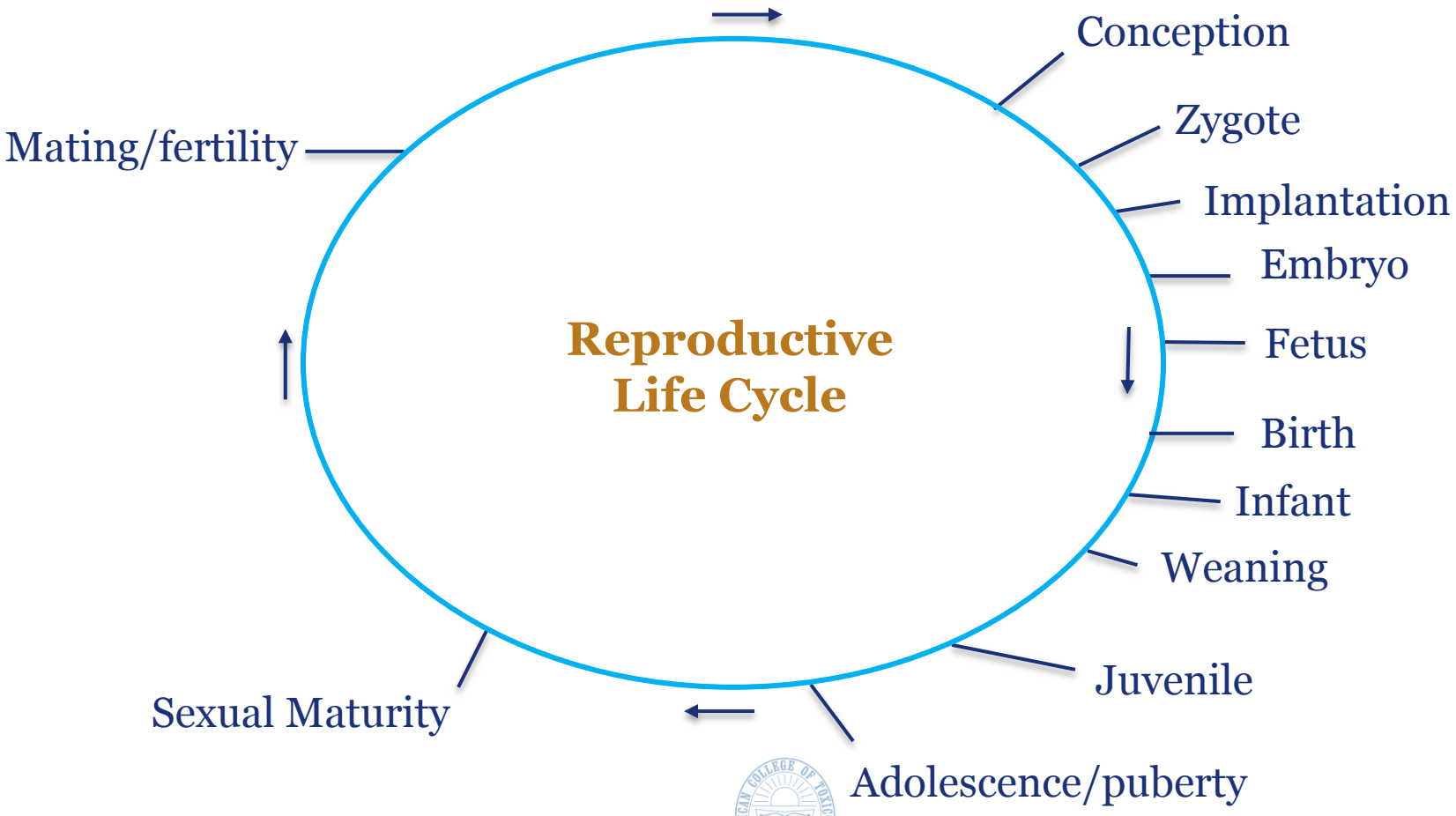
Frequencies of Selected Reproductive Failures

EVENT	FREQUENCY	
	Per 100	Unit
Failure to conceive after one year	10–15	Couples
Spontaneous abortion, 8–28 weeks	10–20	Pregnancies/women
Chromosome anomalies in spontaneous abortions, 8–28 weeks	30–40	Fetuses
Chromosome anomalies from amniocentesis, > 35 years	2	Amniocentesis specimen
Stillbirths	2-4	Stillbirth and live births
Birth weights < 2,500 g	7	Live births
Birth defects	2-3	Live births
Chromosome anomalies, live births	0.2	Live births
Severe mental retardation	0.4	Children to age 15 yrs

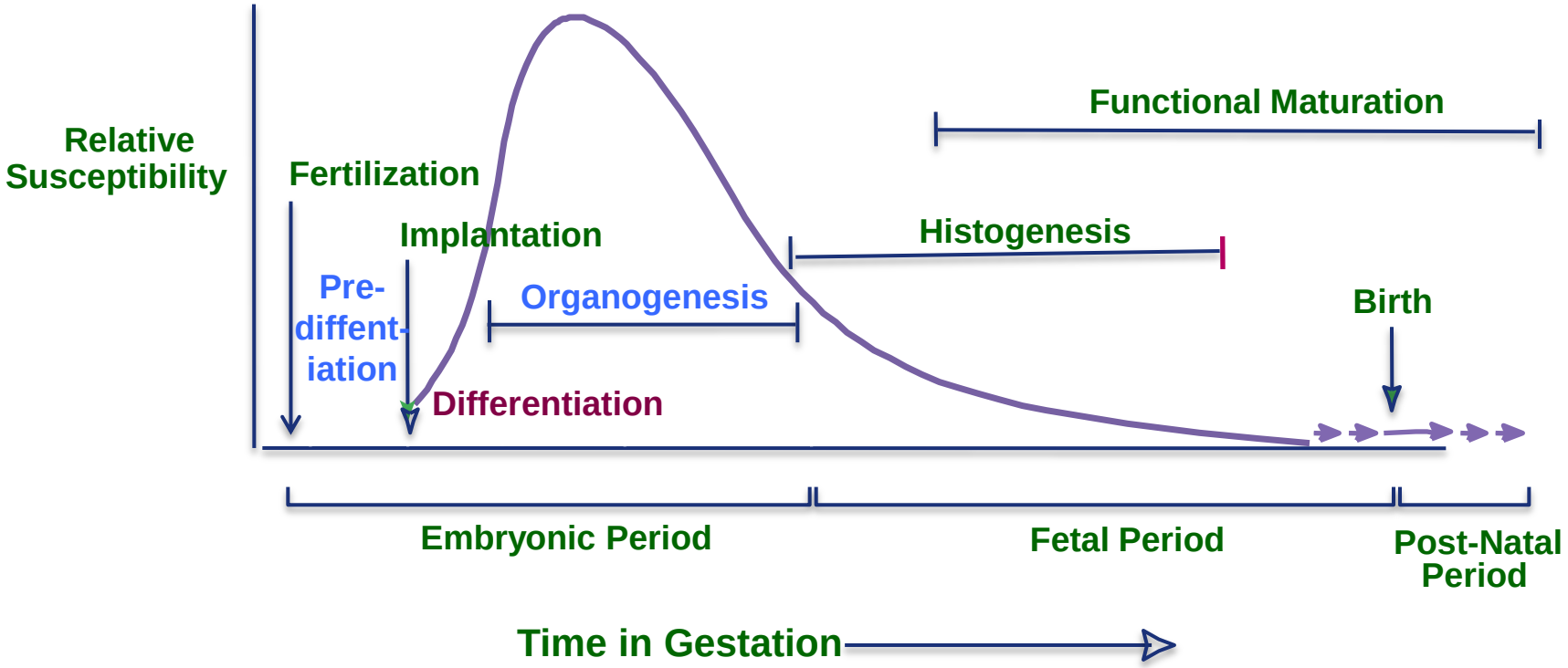
Causes of Birth Defects



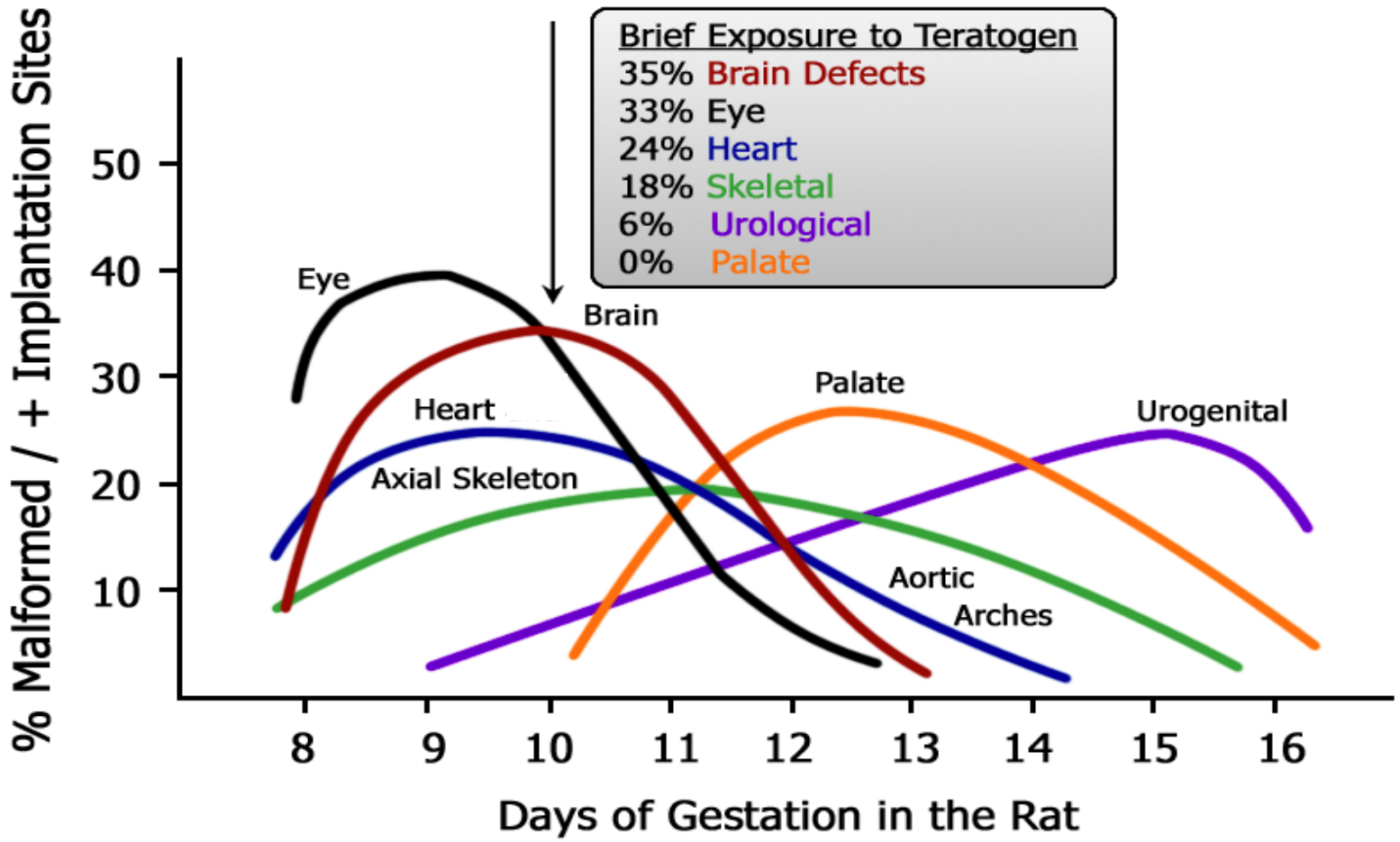
Developmental and Reproductive Stages



Developmental Stages



Hypothetical Patterns of Susceptibility of Fetal Organs to Alterations as a Function of Time



Time of Administration with Various Outcomes

Mouse developmental toxicity after various developmental times following one intraperitoneal dose of 100-120 µm/kg mitomycin -C on GD 6	
GD 7	Embryos smaller but morphologically normal
GD 8	Embryos smaller and morphologically retarded
GD 13	Embryos smaller; defects in 1 litter and reduced litter size
Birth	Pups normal size, increased number of stillborn pups
Weaning	64% mortality, runts, pups with motor defects & tremors
Maturity	Reduced fertility

Source: Snow and Tam, 1979

Species Selection - Advantages of These Species

Guidelines usually require a rodent and non-rodent species (usually rabbit)

<u>Advantages</u> of using the mouse or rat, and rabbit:

- **Cost-effective, large historical control (HC) database, have multiple offspring, high fertility rates, short gestation periods and good suppliers available**
- **Rats are used in repeat dose studies making read across available; reproductive physiology is well understood**
- **Mice use less test article; inbred, transgenic and disease models are available**
- **Rabbits are a nonrodent model, have large fetuses to examine, and semen can be collected for longitudinal assessment**

Species Selection - Disadvantages of these species

Disadvantages of these species:

- Rats have increased susceptibility to sex-related tumors, and to dopamine agonists
- Mice have high basal metabolic rates; may soil their feed; have smallest fetuses to examine; limited blood supply; and are prone to aggregation of congenital malformation clusters over time
- Rabbits consume diets inconsistently; they are prone to abortions; sensitive to GI disturbances (eg., antibiotics should not be tested); are induced ovulators (no estrous cycling); and prone to total resorptions when only 1-2 implants are present

Using the Minipig in DART Studies

Advantages

- Purpose bred; used as non-rodent model
- Multiple offspring (5-6);
- Placenta similar to humans than rodents or rabbits
- Sensitive to known teratogens
- May be species already used in general tox program

Disadvantages

- Uses more test article; different housing requirements
- Spontaneous ovulators; IV dosing difficult; gavage requires restraint
- Sexually mature @ 5 months; C-section GD 108-110
- Limited Historical control data; few labs using the minipig for DART work

Using Nonhuman Primates in DART Studies

Advantages of using NHP

- Excellent model to assess biologics (e.g., proteins) since there usually is less antibody formation
- Multiple sampling sites and tissue volume
- Reproductive physiology closest to humans
- Can do radiographs of fetal development instead of doing C-section

Nonhuman Primates in DART Studies

Disadvantages of using NHP

- Single offspring per pregnancy
- Long gestation period
- Sexual maturity: 3-4 years of age
- Limited historical control database and laboratories with experience and expertise
- Expensive and NHP supplies are limited, leading to smaller group sizes
- Some species (e.g., Rhesus) are seasonal breeders

Route of Administrations

Gavage – known dosage; intubation errors if chemical is irritant or have inexperienced technicians; excessive corn oil can cause diarrhea

Diet – smell and palatability may be a problem; mice nest in feed bins; rabbits won't eat ground feed (must pelletize)

Drinking water – smell and palatability may be a problem; rabbits tend to play with water bottles leading to an overestimate of dose

Injections – chemical irritability at injection site may become a problem; injection volume may be a problem; slow and continuous infusion can cause restraint stress

Inhalation – Feed and water must be removed during exposures; get oral exposure by grooming if whole body inhalation; if nose-only, restraint stress and constant changing of tube size during pregnancy as they grow

Dermal – Oral exposure by grooming if non-occluded; constant changing of chamber or patch size throughout pregnancy

Matings
(Remember the males are not part of the study)

Natural

- **Rodents:** in US labs usually one male to one female
  - Advantage – most common; know which male mated with which female
  - Disadvantage – requires large number of extra males (breeder colony)
- Harem style: mostly in non-US labs; one male to 2 to 4 females
  - Advantage – need fewer males
  - Disadvantage - one male could pass a heritable malformation to multiple females; females impregnated later will have decreased pregnancy rates and litter size

Artificial Insemination (AI) – rabbits only

- Must super ovulation with i.v. human chorionic gonadotropin (hCG)

Vendor Supplied Time-Mated – rats or rabbits

- Advantage – no technical expertise or male breeder colony needed
- Disadvantage – no acclimation period, no gestation day 0 body weights nor feed consumption values

Toxicokinetics in Developmental Toxicity Testing

TK is not required but may be crucial in dose selection

- There is little point in increasing the dose if no increase in blood or tissue levels are obtained
- If the half-life is 2 hours, about 1% of the dose remains 12 hours after bolus dosing, and then you do not have continuous exposure. BID, TID should then be considered
- Sometimes chemical blood levels at the mid-dose are close to the high dose levels due to non-proportional increases in systemic exposure (e.g., metabolic saturation)

Key Guidelines for Developmental Toxicity Testing of Pharmaceuticals and Chemicals

ICH S5 (R2) 4.1.3. The Developmental Toxicity or Embryotoxicity Study (exposure Stages C to D) - Pharmaceuticals

USEPA OPPTS 870.3700 Prenatal Developmental Toxicity Study (exposure Stages B to E or C to E) - Chemicals

OECD No. 414, Guidelines for Testing of Chemicals: Prenatal Developmental Toxicity Study (exposure Stages B to E or C to E) - Chemicals

Defining Developmental and Reproductive Stages

Stage A: Premating to conception

Stage B: Conception to implantation

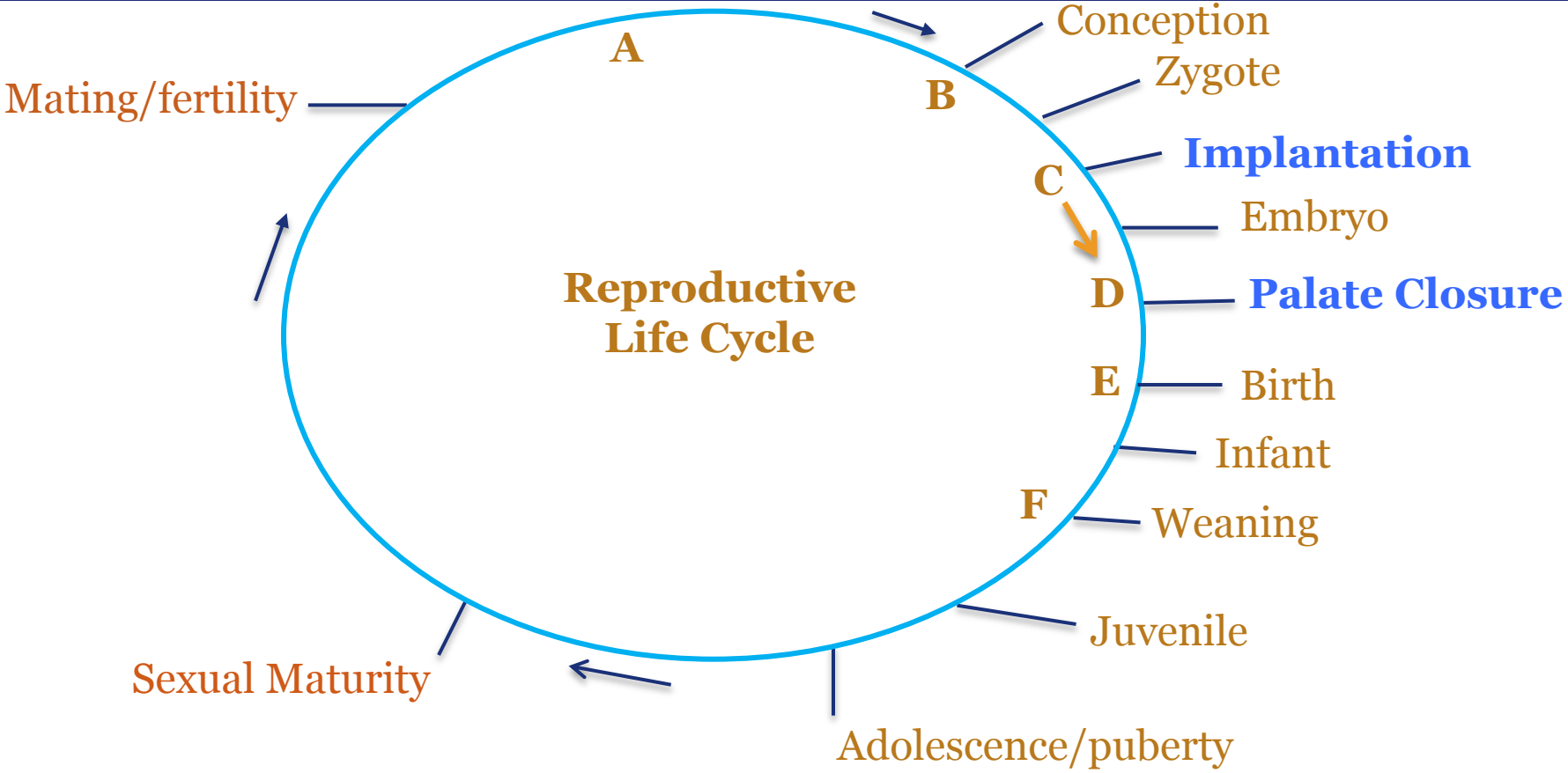
Stage C: Implantation to closure of the hard palate

Stage D: Closure of the hard palate to the end of pregnancy

Stage E: Birth to weaning

Stage F: Weaning to sexual maturity

Reproductive and Developmental Stages



Exposure period for pharmaceutical testing
Stages C to D →

To Do or Not to Do, A Dose Range Finding Study

Preliminary or Range Finding studies are not guideline required, however, if conducted they must be reported

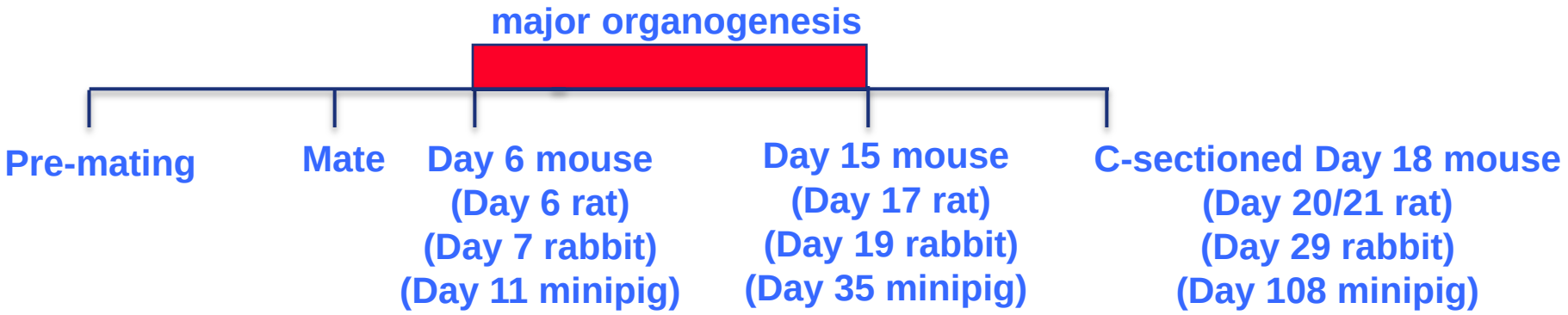
Purpose is to select the highest dose to be given to the dam in the definitive study; need a LOAEL

This may be the first time the test agent is being administered to a pregnant animal or it may be the first time given to this species (i.e., rabbit)

Blood for TK analysis often collected at this time and chemical in the fetus shows it crosses the placenta

Developmental Toxicity Testing of Pharmaceuticals

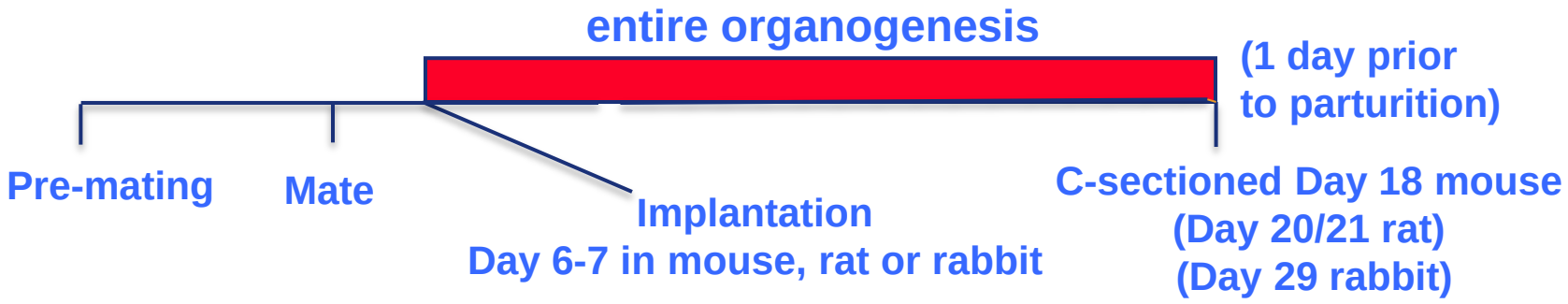
ICH 4.1.3 Guidelines S5A (R2) - (Pharmaceuticals)



- Each exposure level needs to have 16-20 females with live implants so start with 23-25/group for rodents; 22 for rabbits; and 18 for minipigs to evaluate 16 litters
- Assess early and late resorptions, fetal body weights and fetal alterations
- Usually not conducted without a prior dose-ranging finding study

Developmental Toxicity Testing of Chemicals

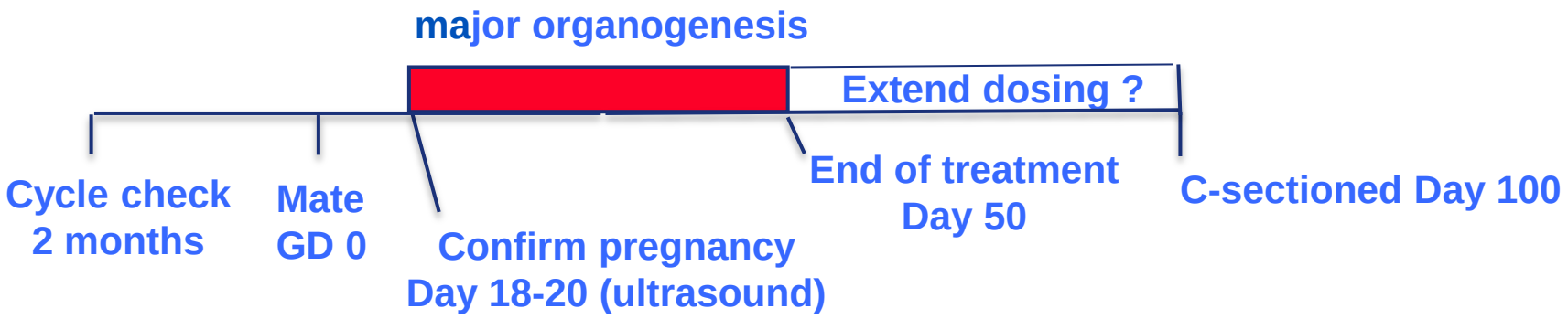
OPPTS 870.3700 and OECD 414 (Chemicals)



Mouse, rat and rabbit prenatal developmental toxicity study

- **Animals are dosed from the day of implantation to one day prior to scheduled Cesarean-section**

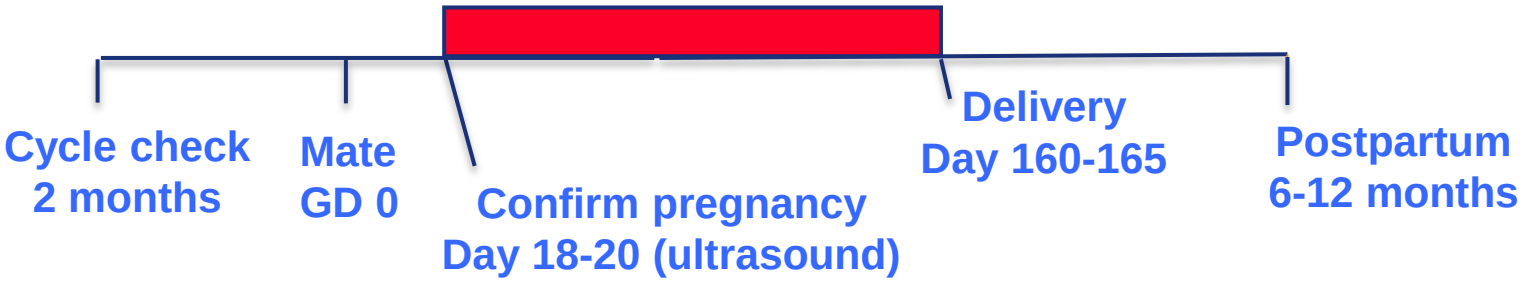
Embryo-Fetal Developmental (EFD) Study in NHP



- Monitor pregnancy monthly throughout pregnancy
- Animals are dosed from the day of confirmed pregnancy and Cesarean-section GD 100
- Collect placenta, maternal blood, cord blood, and amniotic fluid
- Fetal exams: external, visceral, and skeletal exams

Enhanced Pre-and Postnatal (eEFD) in NHP

ICH M3 (R2) and ICH S6 (R1)



- Used to test monoclonal antibodies
- Monitor pregnancy monthly throughout pregnancy
- Animals are dosed from the day of confirmed pregnancy through delivery
- Collect maternal blood/milk, infant blood, physical and neurobehavioral exams
- Infant necropsy: external, visceral, and *in vivo* skeletal exams

Endpoints of Concern in Developmental Toxicity Studies

- **Maternal toxicity**
- **Embryo/fetal death**
  - **Preimplantation loss**
  - **Early resorptions**
  - **Late resorptions**
- **Structural malformations and variations**
  - **External (gross)**
  - **Visceral (Wilson's or Staples')**
  - **Skeletal (Single or double stained)**
- **Growth retardation**
  - **Intrauterine growth (low fetal weight)**
  - **Developmental delays**

Maternal Toxicity vs. Developmental Toxicity

Embryo/fetal effects in the presences of frank maternal toxicity usually elicits less concern from a perspective human risk assessment. But why?

- **The *in utero* progeny are dependent on mother for their physical environment, nutrients, oxygen, and metabolic waste disposal. Thus it is plausible that maternal mediated toxicity may secondarily effect embryo/fetal development**

- **Common findings suspected to be maternal mediated toxicity: supernumerary ribs, rudimentary ribs, wavy ribs, prenatal mortality and retarded fetal growth**

- **There are many different mechanisms by which maternal-mediated toxicity may occur:**

  Stress from MTD, dosing route, feed or water deprivation, restraint, noise and housing conditions

Interpreting Supernumerary Ribs (SNR)

- **In rodent fetuses: SNR (or accessory ribs) are based on length (0.6 mm is dividing point): short (rudimentary) and long (extra)**
  - **Extra ribs are permanent abnormal structures and short ribs are ossification sites that disappear postnatally**
  - **Only the extra ribs increased in frequency in a dose-response fashion to chemicals tested; rudimentary ribs were similar across control and dosage groups**

Rogers et al. 2004, Birth Defects Research, Part B, 71:17-25

Interpreting Wavy Ribs and Delayed Ossifications

- **Wavy ribs**
  - **Not usually relevant to humans**
  - **Usually disappear (look normal) postnatally**
- **Delayed ossification**
  - **Refers to the decrease in mineralization of stained fetal bone**
  - **Biologically reflects growth retardation and often parallels decreased fetal body weight**

Carney and Kimmel, 2007

Key Factors to Interpreting Delayed Ossification (DO)

Insignificant Findings:

- Isolated statistical increases in a few variations but no consistent pattern
- Incidences are within recent historical control ranges

Low Significance Findings (may not be adverse):

- Pattern consistent with other bone delays in phalanges, sternebrae, skull
- Normal cartilage is present
- Associated with maternal toxicity

Findings Warrant Increased Attention:

- Unusual pattern that does not follow normal biological sequence (distal bone development before proximal bone development)
- Specific delays involving long bones that normally are well ossified at term
- Abnormally-shaped cartilage template
- Occur without decreases in fetal body weights
- Occur in the absence of maternal toxicity
- Occur associated with teratogenesis

Carney and Kimmel, 2007

Structural Malformations and Variations

Malformations - structural anomalies that alter general body shape, disrupt or interfere with function and may be incompatible with life

Variations - alterations in structure that have no significant biological effect on the health or body shape and represent slight deviations from normal development

- **Gross Fetal Alterations - Observed grossly at external examination of fetus**
- **Visceral Fetal Alterations – Observed at internal examination of the fetal organs during Staples' fresh examination or Wilson's fixed sectioning**
- **Skeletal Fetal Alterations - Observed grossly at examination, stained (single or double) fetal specimens**

Terminology

- **Many alterations can be named using specific terms found in published glossaries**
  - **Wise, L.D. et al (1997), Terminology of Developmental Abnormalities in Common Laboratory Mammals (Version 1), Teratology 55:249-292**
  - **Makris, S.L. et al (2009), Terminology of Developmental Abnormalities in Common Laboratory Mammals (Version 2). Birth Defects Research Part B, 86:227-327**
  - ***DevTox* Project (http://www.devtox.org) for nomenclature and images and does note some malformations in multiple species**
- **Most are descriptive terms rather than diagnostic, and not interpretive (i.e., they don't distinguish between malformation and variation)**

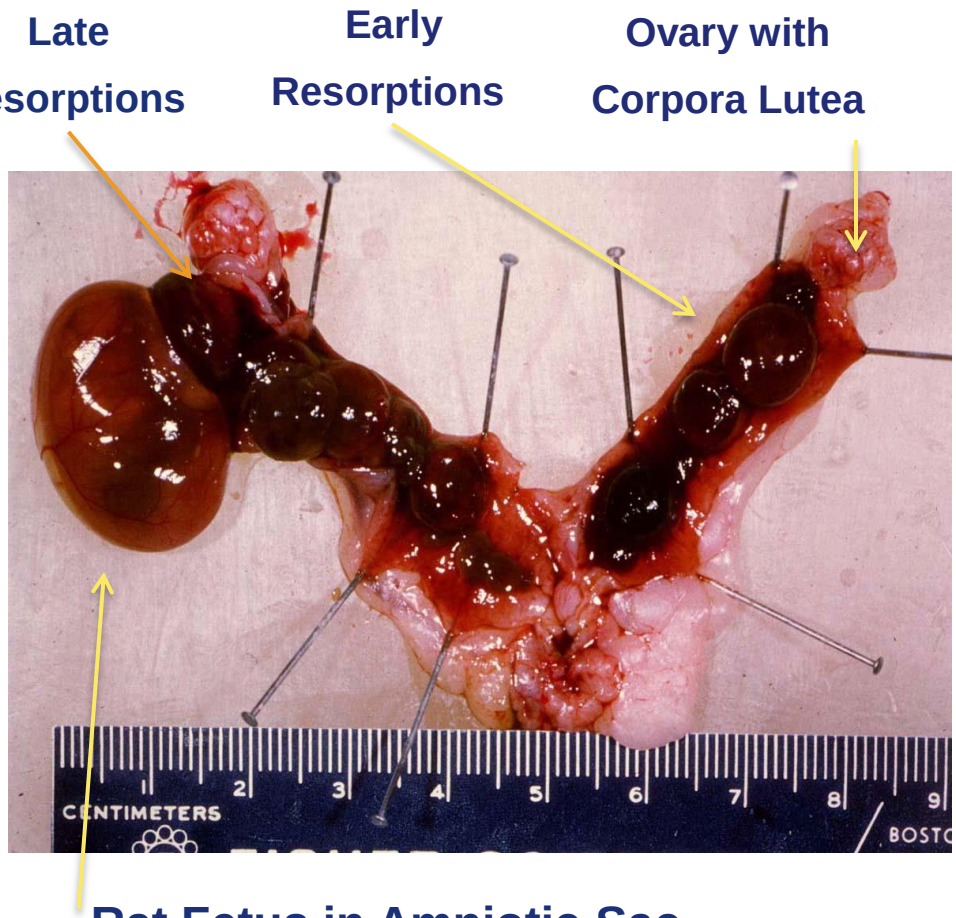
Embryo/fetal death

Early resorption - a conceptus with no evidence of organogenesis

Late resorption - a conceptus with evidence of organogenesis but marked evidence of autolysis (GD 16+ in rat)

Dead fetus - will not respond to stimuli and has no autolysis, while a live fetus will move

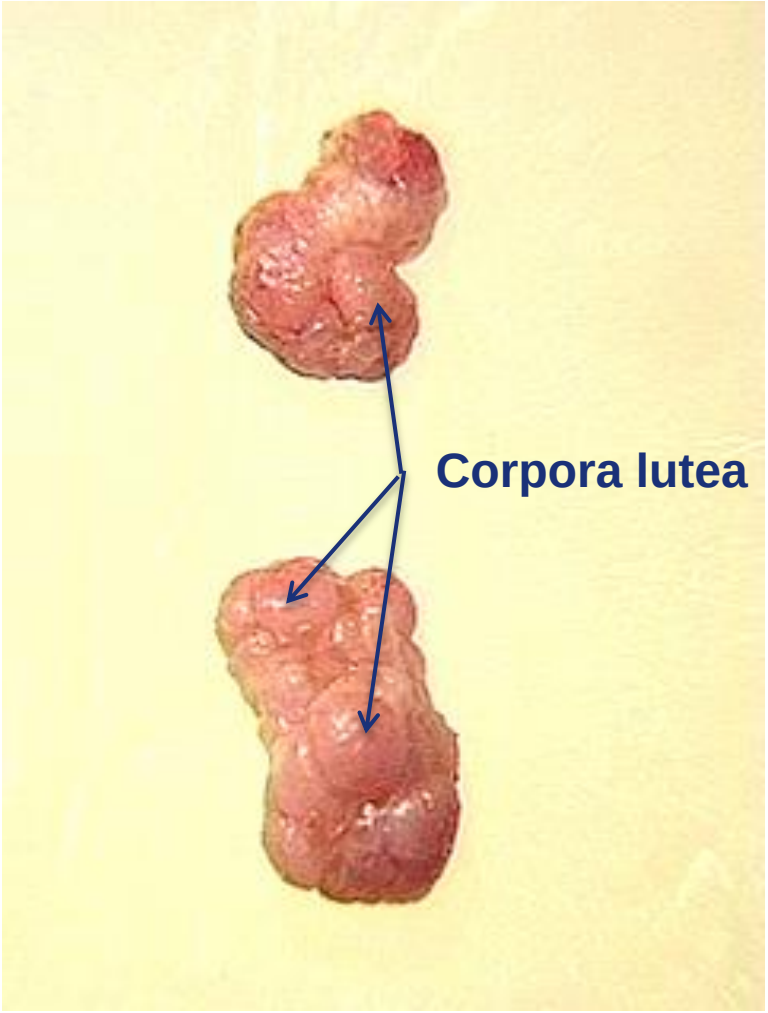
Abortion - interruption of pregnancy before term; rabbits abort while rats usually do not



Rat Fetus in Amniotic Sac

Rat ovaries - Corpora lutea

- Rat ovaries with corpora lutea, appear as round, slightly pink, discrete swellings
- The number or corpora lutea should equal or exceed the total number of implantations
- The corpora lutea count is used in determining the pre- and post-implantation loss



Embryo/fetal Death and Developmental Toxicity

$$\frac{\text{Preimplantation loss}}{\text{litter (\%)}} = \frac{\text{(\# corpora lutea - \# implants) / litter}}{\text{\# corpora lutea / litters}} \times 100$$

Increase rate indicates adverse effect on gametes, fertilizations, zygote, blastula and/or implantation and will result in a reduced litter size.

$$\frac{\text{Postimplantation loss}}{\text{litter (\%)}} = \frac{\text{\# dead fetuses + resorptions / litter}}{\text{\# implants / litters}} \times 100$$

Typically considered an adverse effect when it reaches twice the concurrent controls. An increased rate will also result in a reduced litter size.

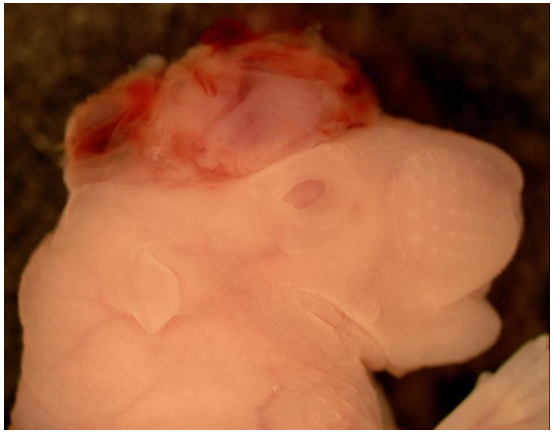
Distribution of Resorptions in Sprague-Dawley Rat Historical Control Data

An increase in the number of dams with 3 or more resorptions is <u>usually</u> a signal of developmental toxicity

Resorptions	# Females	% of Total
0	867	54.4
1	492	30.9
2	177	11.1
3	38	2.4
4	10	0.63
5	2	0.13
6	3	0.19
7	2	0.13
8	1	0.06
9	0	0.00
10	1	0.06
Total	1593	100%

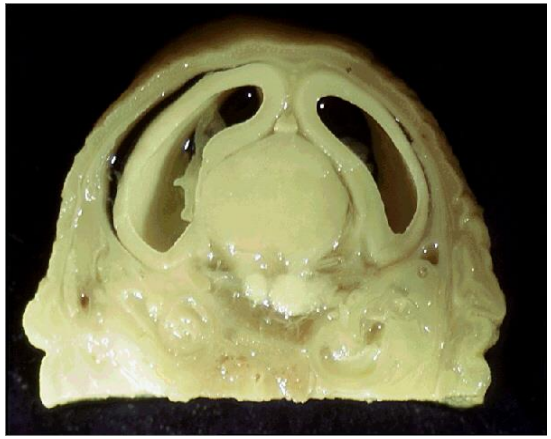
(Historical Control data from one lab and for one year)

Alterations in the General Shape and Development of the Head



Exencephaly – external

Incomplete closure of the cranial vault through which the brain protrudes



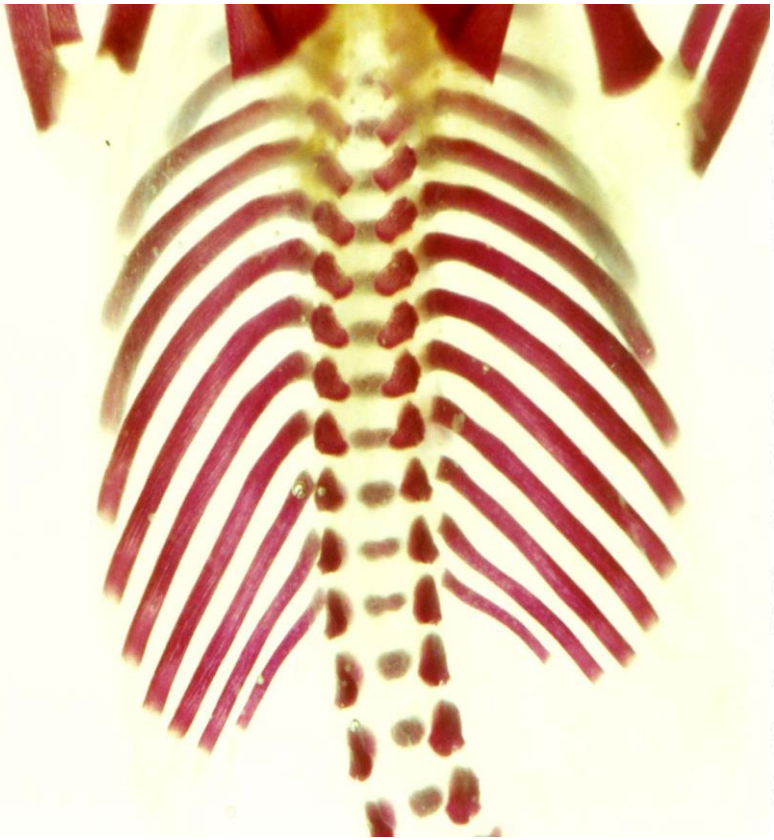
Dilated Ventricles - visceral

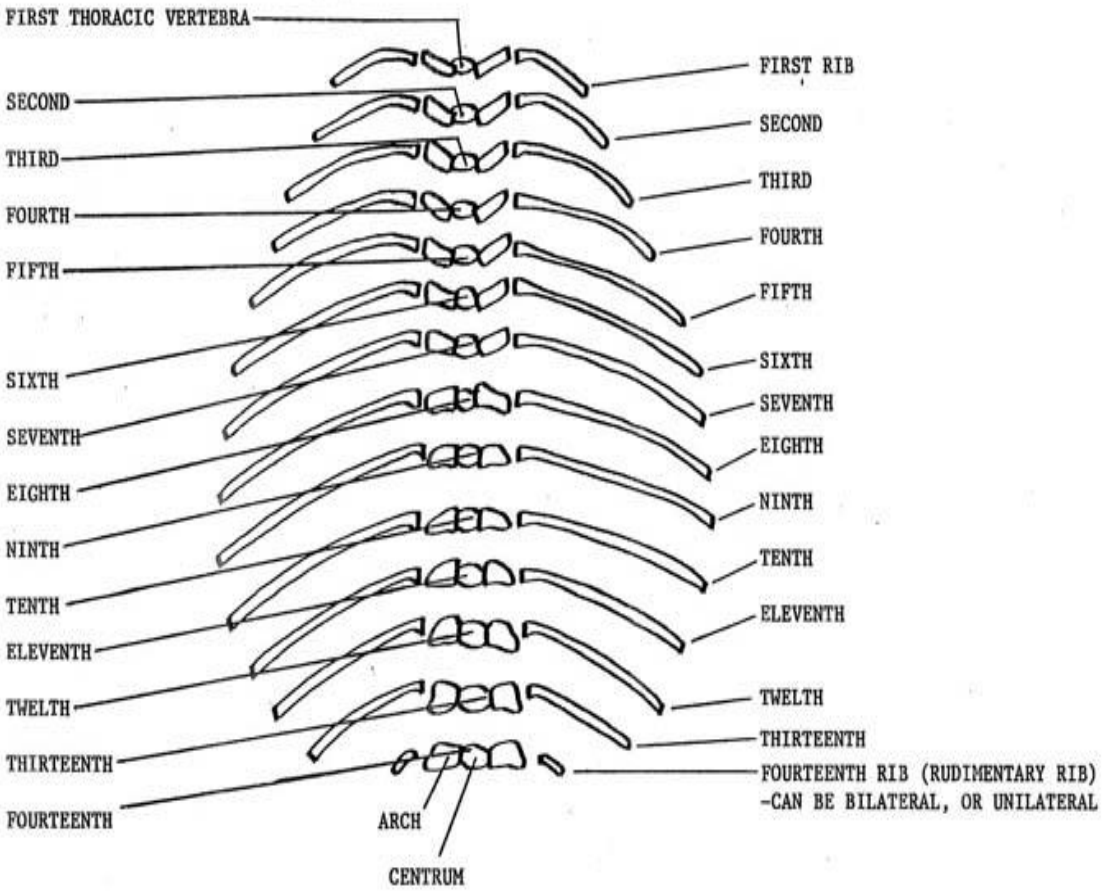
Dilated lateral and third brain ventricles

Skull Unossified - skeletal

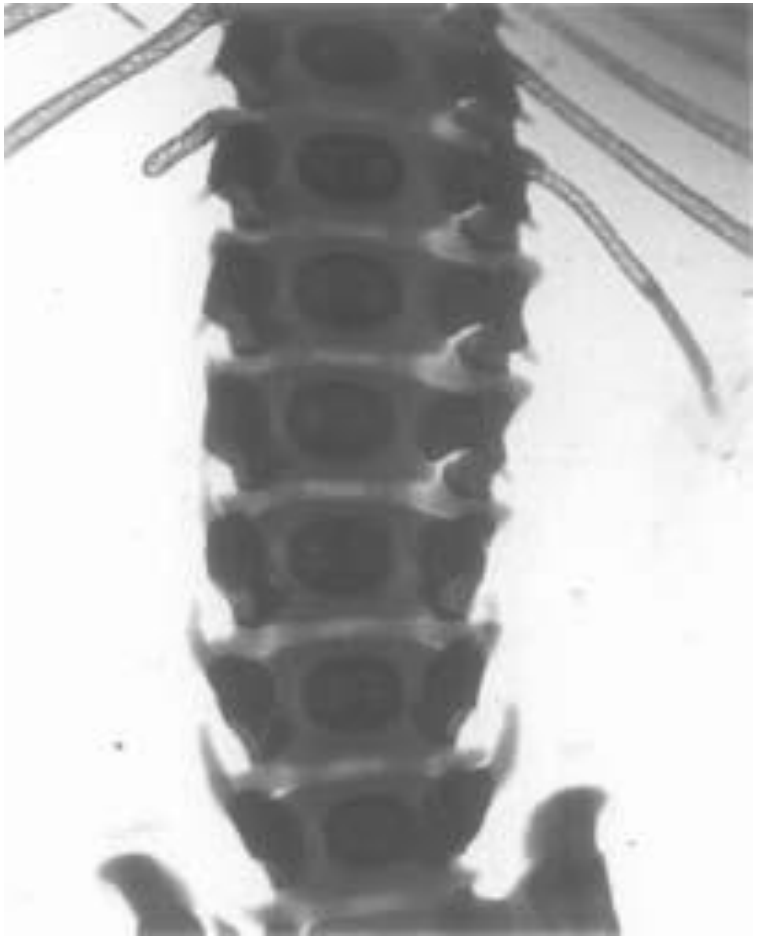
Incomplete ossification frontal bones

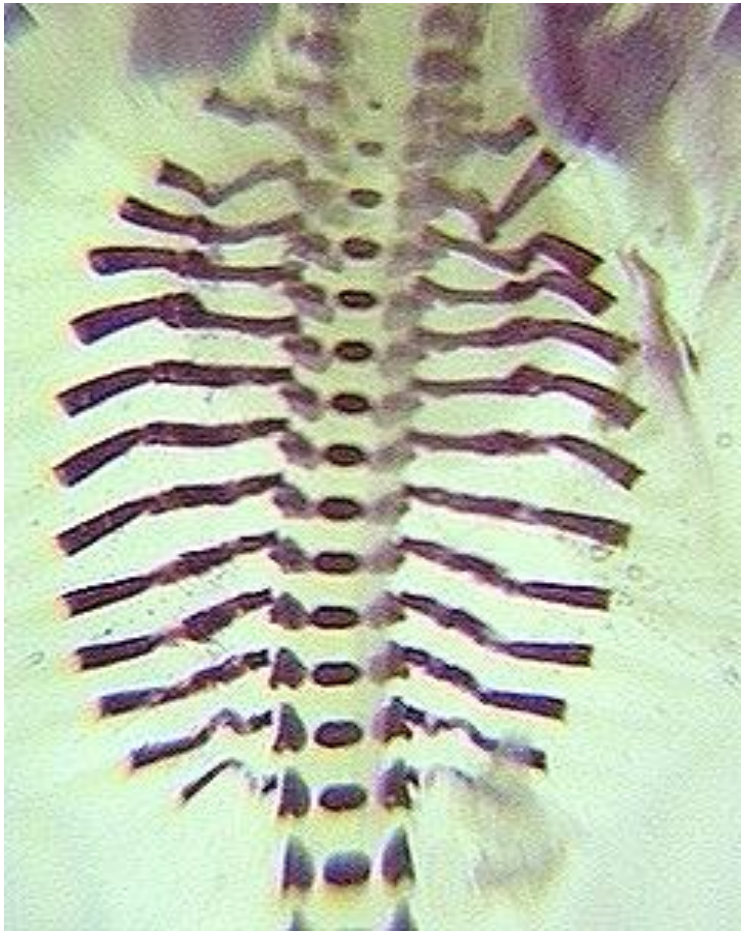
Normal and Supernumerary Rat Ribs (14th)





Mouse Rudimentary Ribs and Rat Wavy Ribs





Endpoints Collected in Developmental Toxicity Study

Ranked by Sensitivity (Most to Least)	
Fetal weights	Maternal gravid uterine weight
- Male	Maternal net body weight
- Female	Maternal feed consumption
Postimplantation loss	Maternal survival
- Early resorptions	Maternal clinical observations
- Late resorptions	Maternal necropsy findings
Fetal viability	Maternal clinical pathology
Fetal malformations	Maternal organ weights
Fetal variations	# Corpora lutea
Maternal body weight	# Implantation sites
Maternal body weight change	Preimplantation loss

Statistical Considerations – Litter Effect

The maternal dam or litter must be the experimental unit of measure because it is the unit that is randomized and the individual fetuses or pups do not respond completely independently

Litter	Resorptions	Implantations	Postimplantation loss (PI)
1	0	13	0%
2	5	10	50%
3	0	16	0%
4	0	14	0%
5	1	14	7%
Sum	6	67	57%

Incorrect Statistics (by implant)

Numerator = 6 = Total no. of resorptions

Denominator = 67 = Total no. of implantations

% PI loss = **9.0%** = Total resorp/total implants

Among-litter viability = Incalculable

Correct Statistics (by litter)

Numerator = 57% = Sum PI loss per litter

Denominator = 5 = Total number of litters

% PI loss **= 11.3%** = Σ PI loss/litter/total litters

Among-litter viability = **21.8%**

Reproductive Toxicity Testing in Animals

Pharmaceuticals

- **ICH 4.1.1. The Fertility and General Reproductive Performance Study (Stages A to C or A to D)**

  Addendum to ICH 4.1.1. Detection of Toxicity to Reproduction for Medicinals

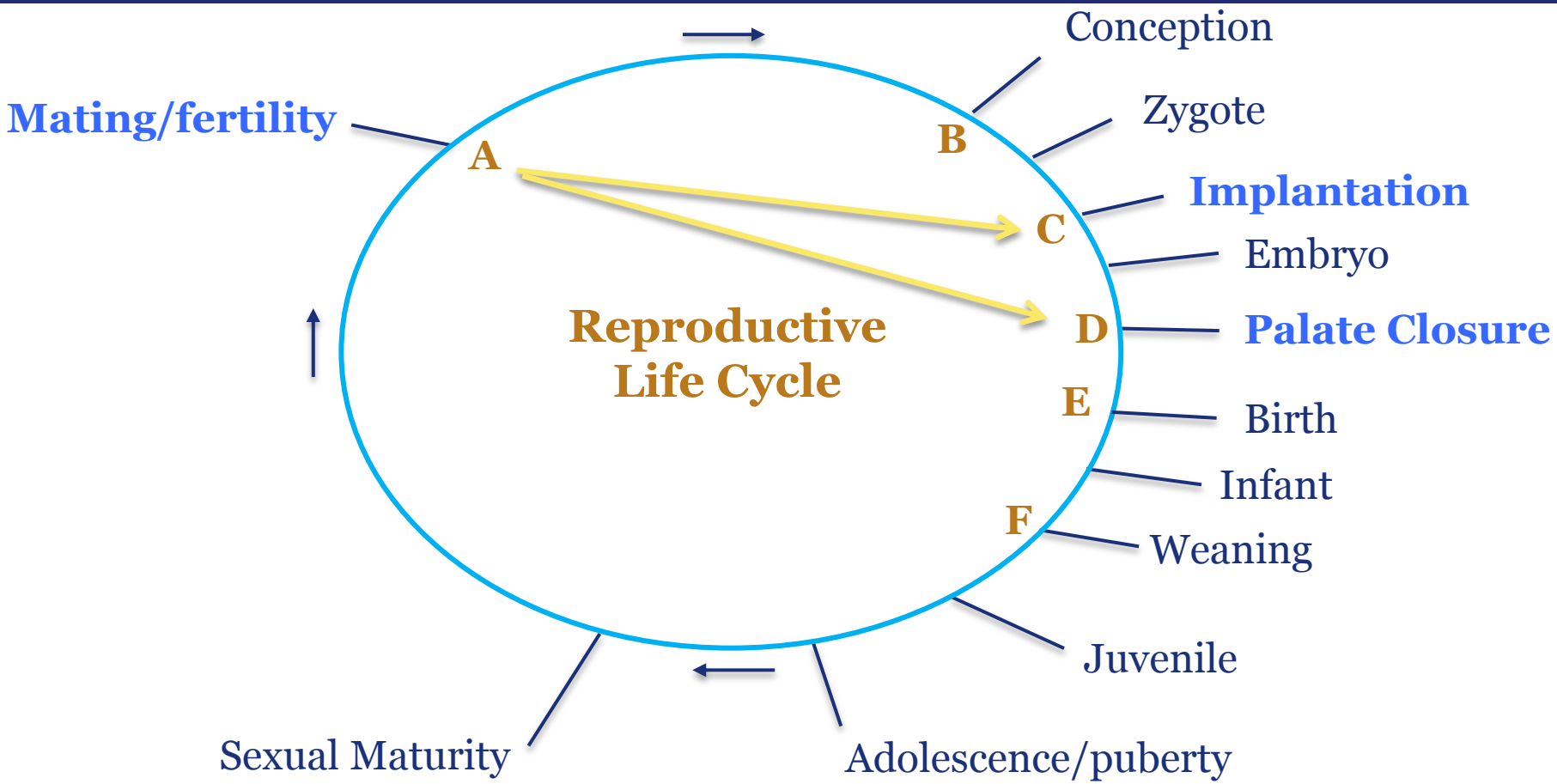
- **ICH 4.1.2. The Prenatal and Postnatal Study (Stages C to F)**

Chemicals – EPA and OECD guidelines are essentially harmonized

- **USEPA OPPTS 870.3800, Reproduction and Fertility Effects (Two-Generation)**
- **USEPA OPPTS 870.3550, Reproduction/Developmental Toxicity Screening Test**
- **OECD #415, One-Generation Reproduction Toxicity Study**
- **OECD #416, Two-Generation Reproduction Toxicity Study**
- **OECD #443, Extended One-Generation Reproductive Toxicity Study**

Note: OPPTS name change to Office of Chemical Safety and Pollution Prevention" or OCSPP but did not affect the Guidelines

Fertility and General Reproductive Performance



Exposure period for fertility testing of a pharmaceutical
Stages A to C or A to D

Reproductive Toxicity Studies for Pharmaceuticals

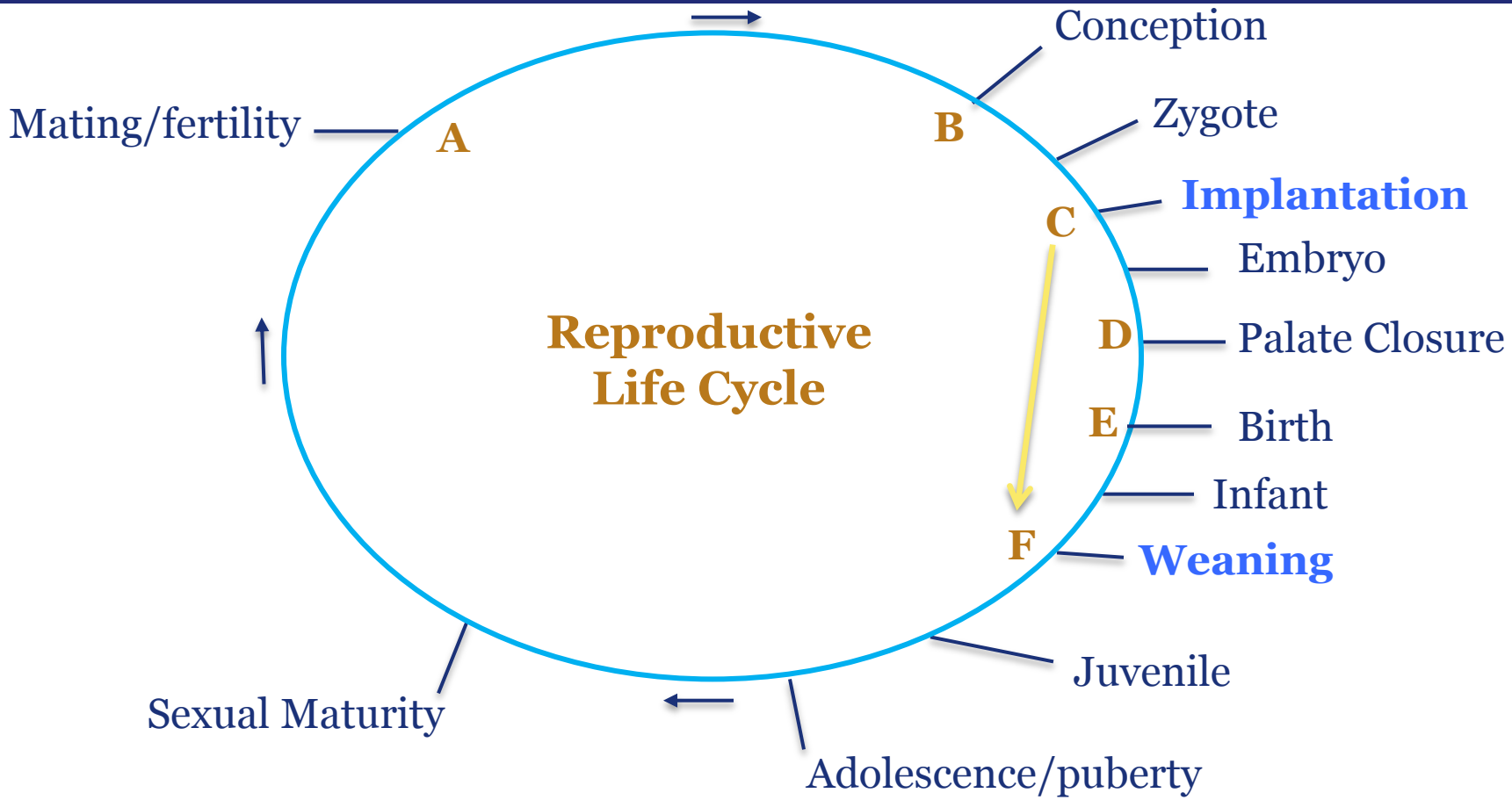
ICH 4.1.1 Guideline S5A (R2) - Choice of Designs

1. **Rat fertility and embryonic toxicity study (Stages A to C)**

2. **Rat fertility and embryo-fetal toxicity study (Stages A to D)**

*Dose 2 weeks premating for males if there is a no effect study of one month or longer, otherwise dose males for 10 weeks and females 2 weeks premating

Prenatal and Postnatal Study

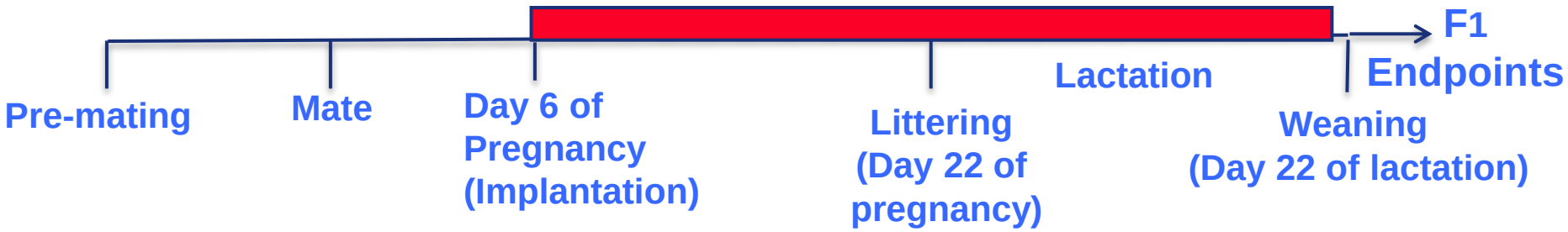


Exposure period for perinatal testing of a pharmaceutical
Stages C to F

Perinatal Reproductive Toxicology

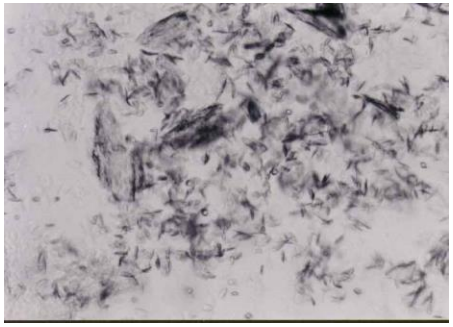
ICH 4.1.2 Guideline S5A (R2)

Rat pre- and postnatal developmental toxicity study (Stages C to F)



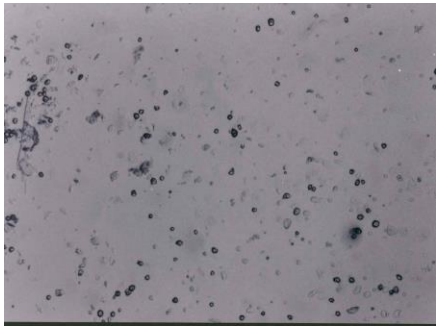
- Typically extended to assess F1 generation to measure pup physical development, developmental landmarks, sexual maturation, sensory functions and reflexes, learning and memory, and mating performance (takes 15 weeks)
- Two animals/sex/litter are used for behavioral testing and sexual performance
- Mated F1 females are C-sectioned GD 15-21, depending on the laboratory

Estrous Cyclicity for Rats

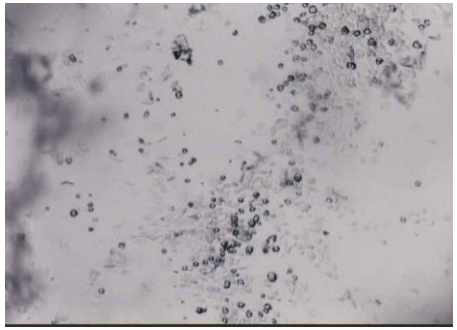


Stage 1: Estrus
10 to 15 hrs

Stage 2: Metestrus
approx 6 hrs



Stage 3: Diestrus
2-3 days



Stage 4: Proestrus
8 to 12 hrs

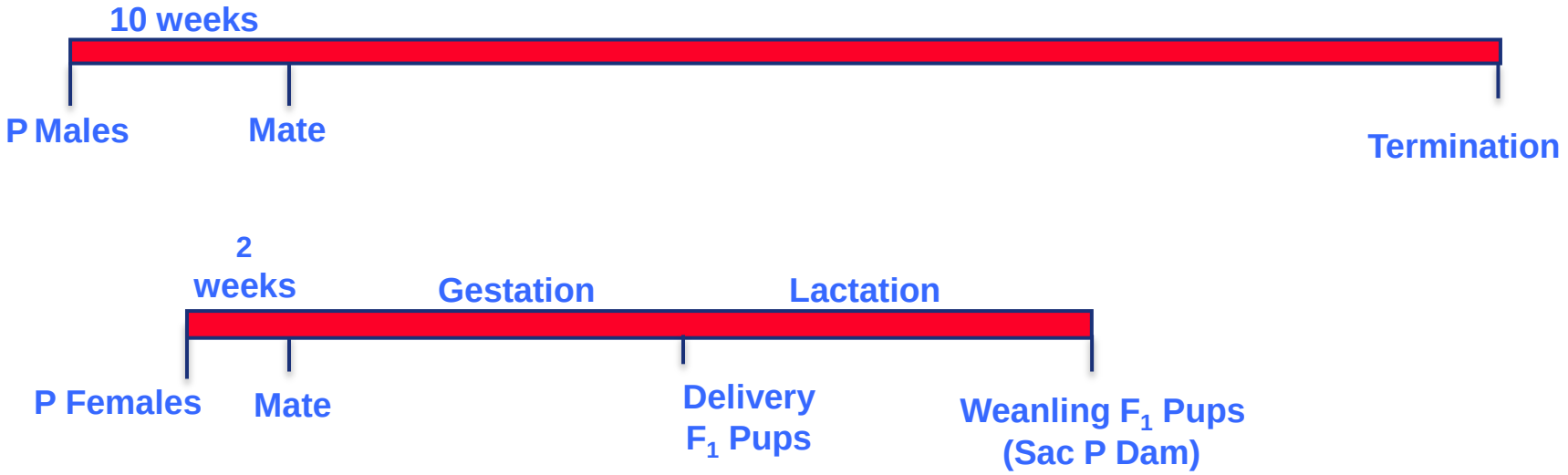
Estrous cycling begins at puberty (approx. 45 days of age in rat; rabbits are spontaneous ovulators)

Normal rat estrous cycle is 4 to 5 days; only receptive for mating the evening of proestrus

Repeated extended cycles or persistent estrous or diestrus may be an indicator of reproductive toxicity or pseudopregnancy

One-Generation Rat Reproductive Toxicology Study

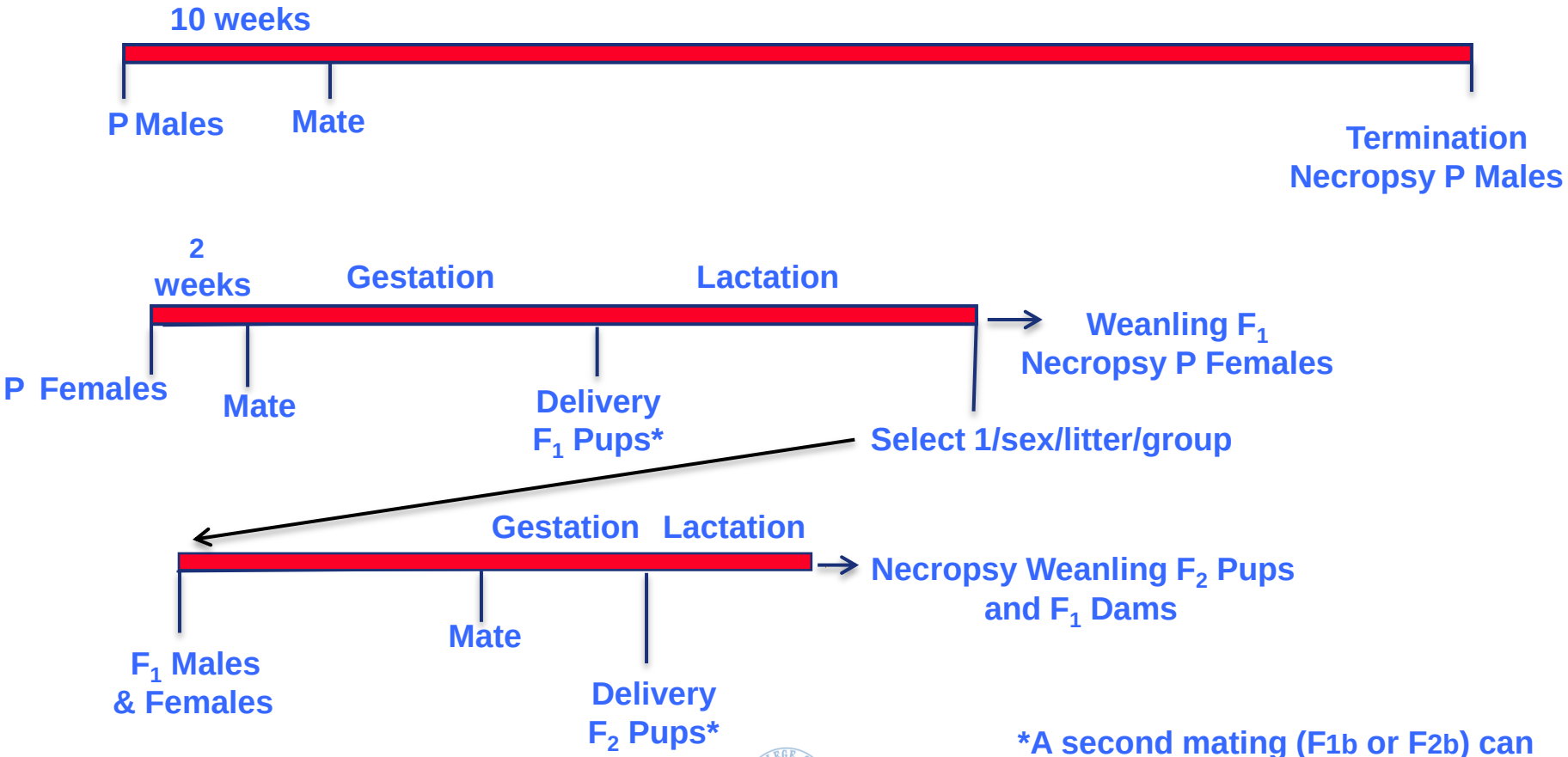
OPPTS 870.3800 and OECD 415 - Chemicals



- **Clinical observations, body weights, feed consumption of all P generation rats**
- **Sex of pups, stillbirths, live births, gross malformations, body weights by sex for all F1 generation pups**
- **Histopathology of sex organs, pituitary and target organs from all P generation rats**
- **Limit Test: 1000 MKD**

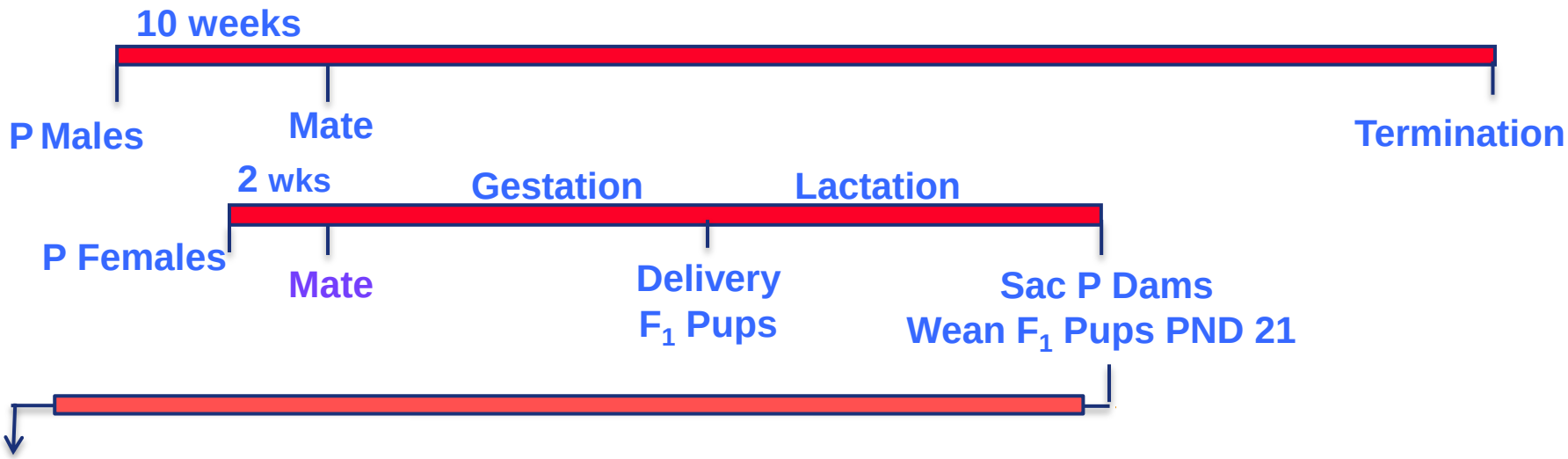
Two-Generation Rat Reproduction Toxicology Study

OPPTS 870.3800 and OECD 416 - Chemicals



*A second mating (F1b or F2b) can be used for a teratology study

Extended One-Generation Rat Reproductive Study



Weaned F_1 Pups	Cohort	Endpoints	Sexual maturation	Age at necropsy
Target is 20 litters per group	1A	Reproductive + general toxicity	yes	13 wks
	1B	Reproductive; mated if triggered from P gen	yes	14 or 20 wks
	2A	Neurotoxicity; neurobehavioral & neuropath	yes	9 wks
	2B	Neurotoxicity; neurohistopath PND 21/22	no	3 wks
	3	Immunotoxicity; PND 56 ± 3	yes	8 wks
	surplus	spares	no	3 wks

Extended One- vs. Two-generation Toxicity Study

Provides more flexible and tailoring of the testing design

Can covers all life stages with better use of the F1 rats

Provides added DNT and DIT information which two-generation study does not

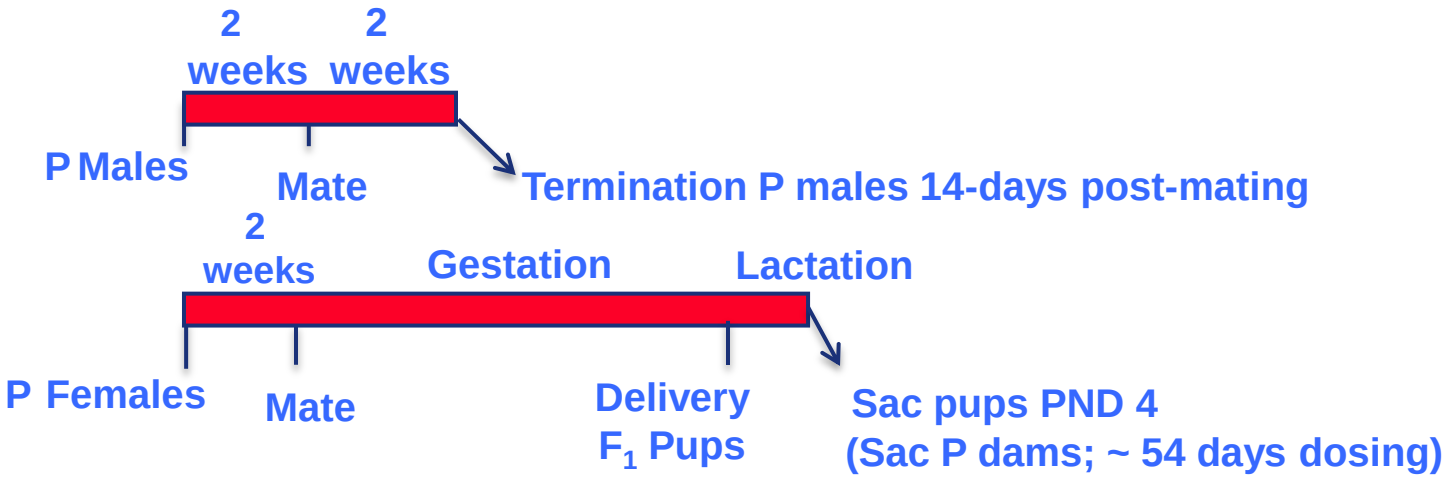
Can replace the stand-alone Developmental Neurotoxicity and Developmental Immunotoxicity studies

Reduced usage of animals (approximately 3000 vs. 5000 rats)

However, cost more than two-generation if DNT and DIT included and has more complexity and therefore more logistical challenges

Reproductive/Developmental Toxicity Screen

OPPTS 870.3550 and OECD 421 - Chemicals



Control and 3 treatment groups; 10 rats/sex/group (expect 8 pregnancies)

Clinical observations, body weights, feed consumption of all P generation rats

Sex of pups, stillbirths, live births, gross anomalies, body weights by sex of all F_1 pups

Weigh testes, epididymides and litters; count corpora lutea, implants, and live pups by sex (PND 1 & 4)

Histopathology of ovaries, testes (staging), epididymides and target organs from control and highest groups

Endpoints Collected in Reproductive Toxicity Study

Ranked by Sensitivity (Most to Least)	
Litter size/live birth index	**Duration of gestation**
Neonatal growth	Gestation Index
Neonatal survival indices	**Sexual landmarks**
Prenatal lethality	**Functional landmarks**
Sperm quality	CNS maturation
Reproductive organ weights	**Learning and Memory**
Pathology of reproductive organs	Nesting/**Nursing behavior**
Estrous cyclicity	Sexual behavior
Precoital interval	Sex ratio of offspring
Mating/Fertility indices	Oocyte quantification

Bold blue endpoints may be assessed in humans

Viable Litter Size and Live Birth Index

$$\text{Litter Size (LS)} = \frac{\text{\# of pups born (live + dead)}}{\text{\# of dams delivered}}$$

$$\text{Live Litter Index} = \frac{\text{Total viable pups at birth}}{\text{\# total pups born}} \times 100$$

- Stable indices and are frequently the most sensitive indicators of reproductive toxicity
- Very important to examine litters ASAP after birth to assess stillbirths and prevent cannibalism
- Decrement of ≥1.5 pups per litter from control values is usually an indication of reproductive toxicity
- Decrease in LS with no decrease in fertility or PI-loss, is consistent with a dominant lethal mutation effect

Neonatal Growth

Reduced birth weights and pup body weight gains are good indicators of reproductive toxicity

Pup weights are dependent on litter size, pup sex, birth weight and nursing ability

Reduced pup weight gains may be indirect, through decreased nursing or milk production by the dam

If the dietary or drinking water exposure route is used, reduced pup body weight gains can result from direct consumption of the chemical by the pups postnatal days 14 to 21

If exposure is by whole body inhalation route, the removal of feed for 6 hr/day generally causes a 20% reduction in pup body weight gains compared to a study using gavage administration

Neonatal Survival Indices

$$\text{Viability Index}_{\text{PND 4, 7, 14 and 21 (\% per litter)}} = \frac{\Sigma\,[\text{Total number of live pups/litter}]\ (\text{at timepoint})}{\text{Number of pups born/litter}} \times 100$$

- Standardization of litter size (culling) to 8 or 10 at PND 4 is an option in all guidelines; US usually does and Europe usually does not cull; it reduces the variance of pup weights due to litter size

- Total litter loss occurs infrequently in rats (<1%); more than one total litter loss per group is usually an indicator of reproductive toxicity

Sperm Quality - Motility

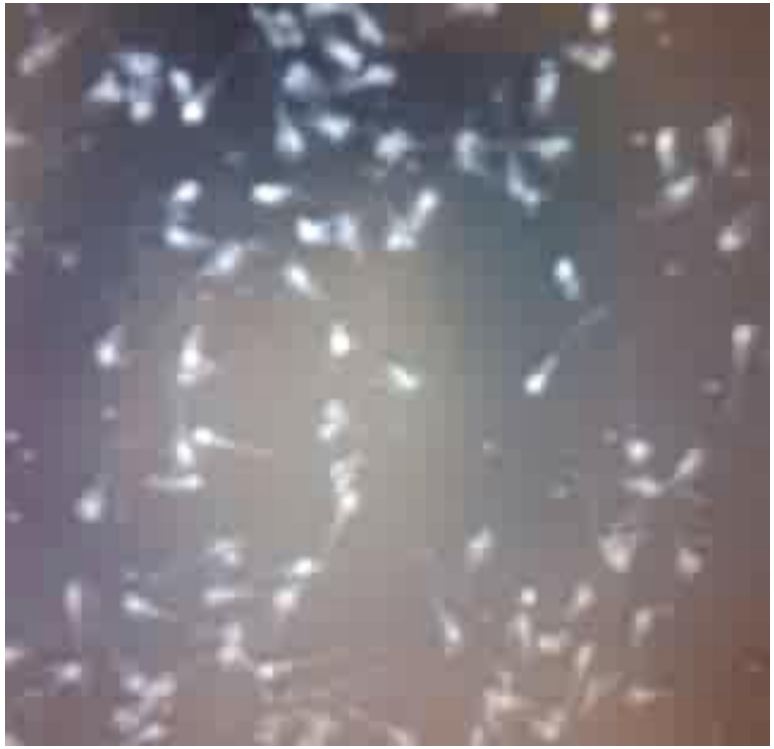
Sperm are collected from the epididymis or vas deferens and are usually measured with a computer-assisted sperm analysis (CASA) system

These sperm are not fully mature and are not accompanied by secretory fluids. Thus these measures will not reflect motility of ejaculated semen samples

Ejaculated semen are often collected from rabbits, dogs, non-human primates, and humans

- **advantages are longitudinal assessment of sperm quality**
- **pretest baseline measurements can be collected**
- **reversibility of an effect can be evaluated on the same animal**

Sperm Motility Assessment



- % Motility
- Path velocity (VAP)
- Progressive Velocity (VSL)
- Curvilinear Velocity (VCL)
- Straightness (STR)
- Linearity (LIN)

Sperm Quality – Count/Concentration

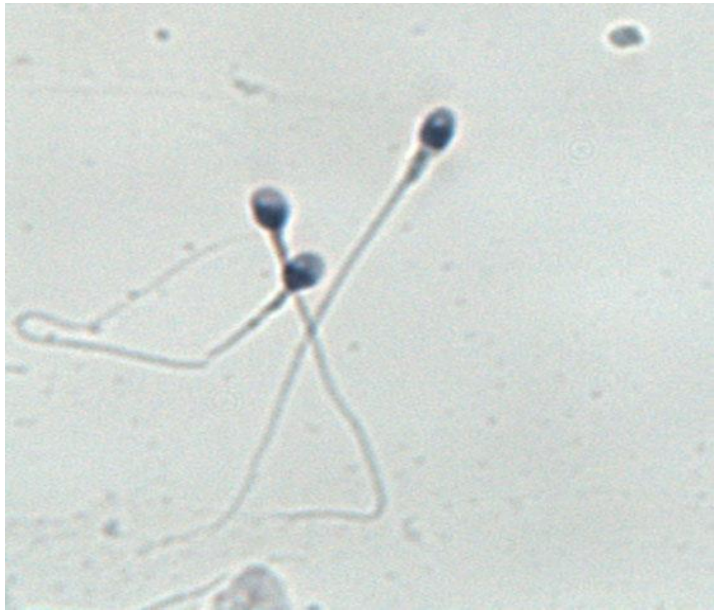
Concentration of spermatozoa is measured by homogenizing the testis or epididymis and counting the number of homogenized-resistant sperm. The concentration is then normalized to the weight of the tissue used.

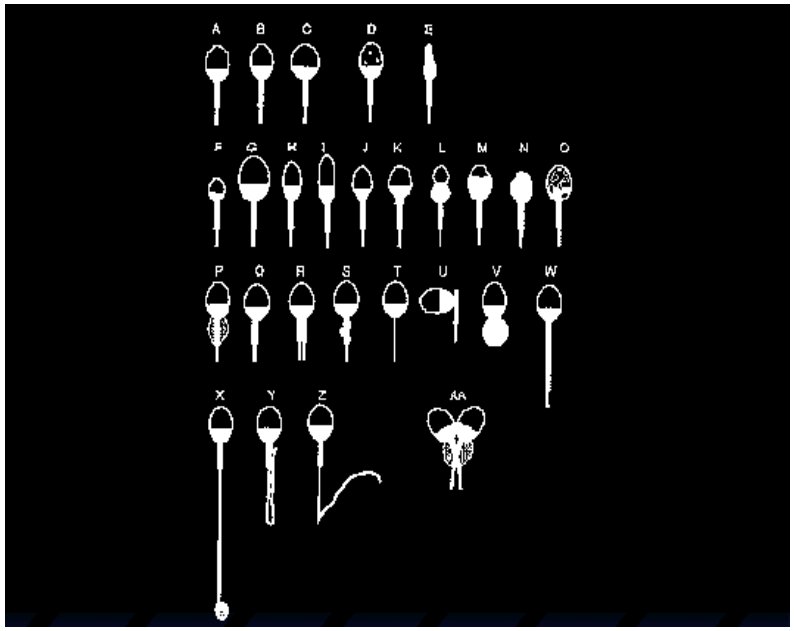
- **Separate counting of sperm in the testis and epididymis would be needed to determine the site of the insult**
- **If the insult occurs during spermatogenesis, both testicular and epididymal counts would be reduced**
- **If testicular sperm counts are normal and epididymal counts are reduced, sperm maturation is most likely affected**
- **Frequently, reductions in sperm concentration are accompanied by reductions in sperm motility and reduced reproductive organ weights**
- **Large reductions in rat sperm concentrations can occur without affecting fertility**

Sperm Quality - Morphology

Sperm morphology is a qualitative determination using light microscopy

Abnormalities of the head, midpiece and tail of the sperm are recorded





Male Reproductive Histopathologic Findings

Most sensitive indicators of toxicity comes from the testes, particularly if response is dose-related

It requires about 63 days for spermatogonia to mature to a spermatozoa in S-D rats

Presence of debris and sloughed cells in the epididymides is usually an indicator of testicular toxicity

Rarely are adverse reproductive effects found in the prostate or seminal vesicles

Female Reproductive Histopathologic Findings

- Increases in follicular atresia, oocyte toxicity or altered corpus luteum formation should be considered an adverse reproductive effect
- Increases in uterine endometrial hyperplasia, hypoplasia or aplasia should be considered an adverse reproductive effect
- Increases in oviductal hyperplasia or hypoplasia should be considered an adverse reproductive effect
- Damaged cells in the anterior pituitary (controls gonadotropin and prolactin production) should be considered an adverse reproductive effect
- Mammary tissue is highly endocrine dependent; significant lesions or changes in the quality or quantity of milk production should be considered an adverse effect

Pregnancy Index and Precoital Interval

$$\text{Pregnancy Index} = \frac{\text{Number of pregnant females}}{\text{Number females with confirmed matings}} \times 100$$

- Pregnancy index measures the female's potential to get pregnant
- Same factors that can reduce the female mating and fertility indices can reduce the female fecundity index

Precoital Interval - or 'mean time to mating' is the average number of days after initiation of cohabitation to time of mating

- An increased precoital interval length suggests impaired sexual behavior or abnormal estrous cyclicity

Male Mating and Fertility Indices

$$\text{Male Mating Index} = \frac{\#\text{ of males confirmed mating}}{\#\text{ of males cohabitated}} \times 100$$

- Confirmation of mating is usually presence of copulatory plug or sperm in vaginal smear
- Can be reduced by physical impairment, acute toxicity, hormonal imbalance or effects on libido

$$\text{Male Fertility Index} = \frac{\#\text{ of males impregnating a female}}{\#\text{ of males cohabitated}} \times 100$$

- Measures the ability of males to produce sperm capable of impregnating a female
- Same factors that can reduce the male mating index can reduce the male fertility index

Female Mating and Fertility Indices

$$\text{Female Mating Index} = \frac{\#\text{ of females mating}}{\#\text{ of females cohabitated}} \times 100$$

- Measures the ability for females to mate (including mating behavior)
- Can be reduced by physical impairment, acute toxicity, hormonal imbalance or alterations affecting libido, and estrous cycle disruption

$$\text{Female Fertility Index} = \frac{\#\text{ of pregnant females}}{\#\text{ of females cohabitated}} \times 100$$

- Measures the percent of copulations that result in pregnancies
- Same factors that can reduce the female mating index can reduce the female fertility index

Gestation Index

$$\text{Gestation Index} = \frac{\text{\# of females with live born}}{\text{\# of females with evidence pregnancy}} \times 100$$

- This index measures the female's ability to maintain pregnancy, based on having delivered at least one live pup
- The gestation index is not a sensitive indicator of reproductive performance because all litters are treated as having equal biological significance regardless of litter size

Sexual Maturation Landmarks in Rats

Preputial Separation

- Onset is considered sexual maturity in male rats
- Examination begins around PND 35 to 38

Vaginal Patency

- Onset is considered sexual maturity in female rats
- Examination begins around PND 25 to 28

Developmental Landmarks in Rats

Developmental landmarks are used to assess pup postnatal development and maturation – looking for patterns of delays

Pinna Unfolding

- Pups are examined for unfolding/detachment of pinna (earflap)
- Each pup is examined daily beginning PND 1 until criterion of either side is met for all pups in that litter

Hair Growth

- Pups are examined for appearance of hair
- Each pup is examined daily beginning PND 1 until criterion of any bristles is met for all pups in that litter

Incisor Eruption

- Pups are examined for incisor eruption
- Each pup is examined daily beginning PND 7 until criterion of any eruption is met for all pups in that litter

Developmental Landmarks in Rats

Eye Opening

- Pups are examined for any membrane break between in eyelids
- Each pup is examined daily beginning PND 10 until criterion of either eye is met for all pups in that litter

Nipple Retention

- Males are examined for presence of nipples
- Each pup is examined daily PNDs 11 through 13 until criterion of no nipples present is met for all male pups in that litter

Testes Descent

- Males are examined for presence of one or both testes in the scrotum
- Each pup is examined daily beginning PND 19 until criterion of testes in the scrotum is met for all male pups in that litter

Behavior and Reflex Testing in Rats

Surface-Righting Reflex

- Pups are examined for regaining the normal position after being placed on its back
- Each pup in a litter is examined daily beginning PND 1 until criterion of surface righting is met within 5 seconds

Hind Limb Placing Reflex

- Pups are examined for raising and placing a suspended foot on a thin rod
- Each pup in a litter is examined daily beginning PND 14 until criterion is met within 5 seconds

Cliff Avoidance Behavior

- Pups are examined for crawling away from an edge of a flat surface (cliff)
- Each pup in a litter is examined daily beginning PND 1 until criterion of cliff avoidance is met within 10 seconds

Behavior and Reflex Testing in Rats

Pinna Reflex

- Each pup in a litter is examined daily beginning PND 13 until criterion of any ear movement is made when lightly touched

Negative Geotaxis Test Behavior

- Pups are examined for ability to change from a downward to upward orientation
- Each pup in a litter is placed on a 30° angle beginning PND 7 until criterion of rotating 180° in 60 seconds

Auditory Startle Reflex

- Pups are examined for a sudden flinch or jump following an auditory stimulus
- Each pup in a litter is examined beginning PND 10 for auditory startle

Behavioral or Reflex Testing in Rats

Air Righting Reflex

- Pups are examined for the ability to land on all four paws when invertedly dropped
- Each pup in a litter is examined daily beginning PND 14 until criterion of landing is demonstrated

Forelimb Grip Test Behavior

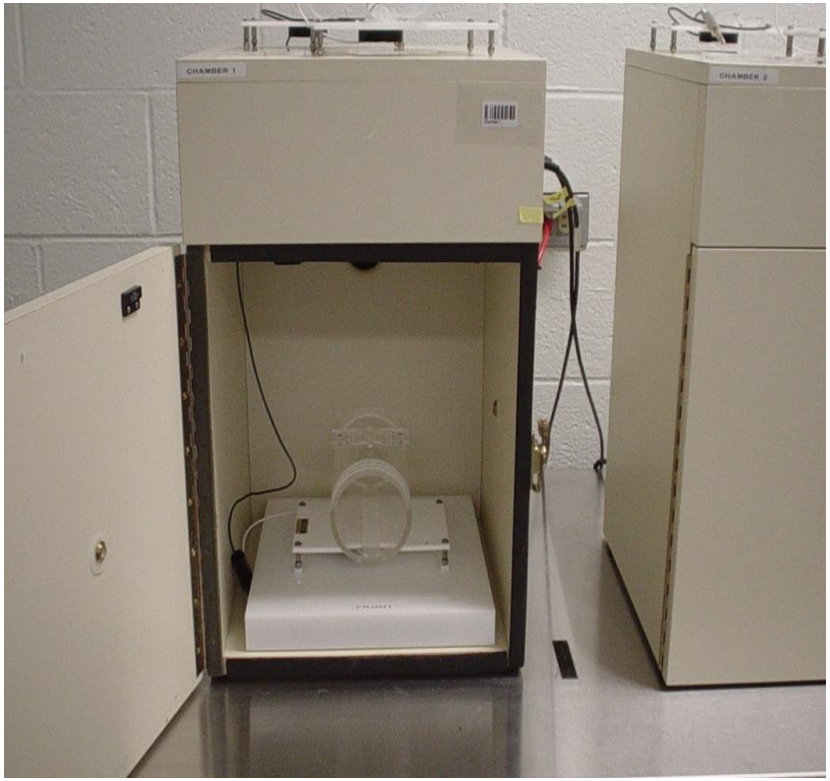
- Pups are tested for forelimb grip by their grasping a thin rod
- Each pup in a litter is tested on PND 21 until criterion of hanging on for 1 second

Pupil Constriction Reflex

- Pups are examined for direct pupillary constriction of both eyes in response to a light beam
- Each pup in a litter is examined on PND 21 until criterion of each eye constricting

Auditory Startle Response

- Animal is placed atop a force transducer in a sound-proof chamber
- The force is applied by the startled animal is measured after a high-pitched noise
- Habituation is assessed PND 21 or 22 and again PND 60 or 61 for latent alterations
- Deficits in peak response should be correlated with deficits in motor activity and grip strength



Locomotor Activity in Rats

- Usually an open field area in a four sided black plastic box with a series of infrared photobeams near the base. Beam breaks are considered locomotor activity

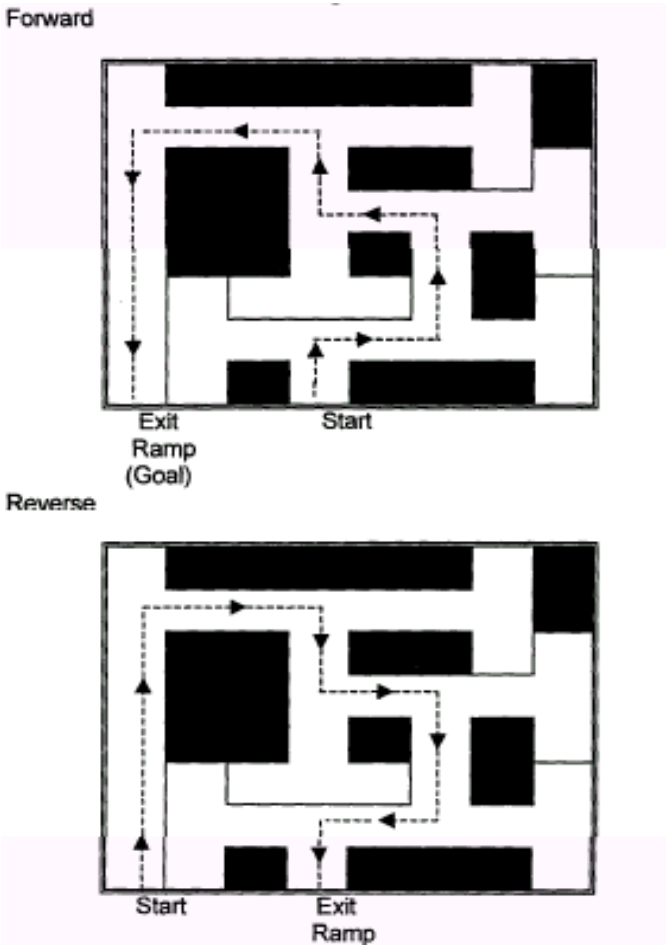
- Assessed PNDs 13, 17 and 21 for ontogeny of habituation and again PNDs 60 to 61 for latent alterations. On PND 13 there should be very little change in total across test intervals. Between PNDs 17 and 21, the first time intervals should have the most activity and gradually decreasing (habituation curve)

- A delay in normal pattern of motor activity development correlating with changes in age- or weight-sensitive parameters should be considered a developmental effect, but if they don't correlate with these parameters, should be considered a neurological effect

Learning and Memory

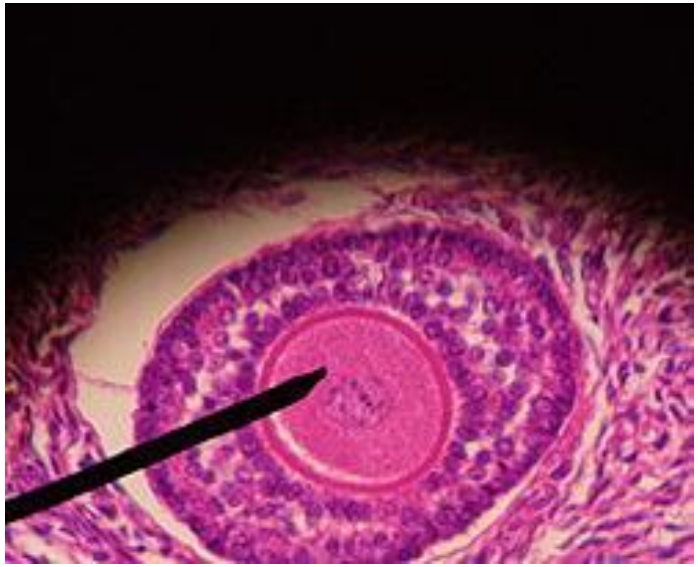
Accepted models are Biel or Morris water mazes, and active or passive avoidance. Each animal is tested for acquisition of learning and memory at a single stage of development, PND 22 or 62.

Day	Number of Trials	Path
1	4	Straight Channel
2	2 (Trials 1 and 2)	Path A (Forward)
3	2 (Trials 3 and 4)	Path A (Forward)
4	2 (Trials 5 and 6)	Path B (Reverse)
5	2 (Trials 7 and 8)	Path B (Reverse)
6	2 (Trials 9 and 10)	Path B (Reverse)
7	2 (Trials 11 and 12)	Path A (Forward)



Oocyte Quantitation

- The ovarian follicle is composed of the oocyte, granulosa cells and the cells of the theca layers (basement membrane)
- Three types of ovarian follicles can be quantified: small or primordial, growing, and antral; many laboratories combine the primordial and growing follicles counts
- Counting antral follicles is problematic because they are 100-550 um in diameter and are often counted multiple times; therefore not often quantified
- Animals that are anovulatory are often recorded as having increased numbers of primordial and growing follicles because they are easier to see (no large antrals to block their view)



Human ovarian follicle

A significant decrease in ovarian follicle count is usually considered an adverse effect because recovery is not possible

Thank you for your participation in the American College of Toxicology Webinar on

"Understanding Developmental and Reproductive Toxicity Studies"

Are there any questions?

Thank you for your participation in the American College of Toxicology's Webinar!

We hope to see you at …