Developmental Toxicity of Perfluorinated Compounds: A Voyage from Animal Studies to Transfected Cells.

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Disclosure Slide

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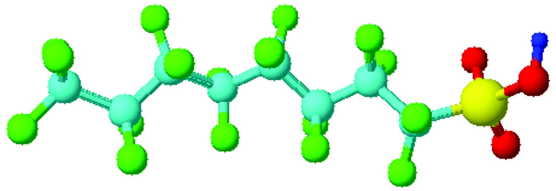
Perfluoroalkyl acids (PFAA)

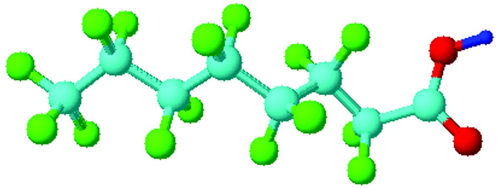
A family of organic fluorochemicals and their derivatives

Perfluorooctane Sulfonate (PFOS)



Perfluorooctanesulfonic Acid

Perfluorooctanoic Acid (PFOA)



Perfluorooctanoic Acid

- Wide industrial and household applications
  - Coatings for paper, fabrics, fire-fighting foams, insecticides, electronic etching baths, and other uses
- Bioaccumulate, environmentally stable
- Global distribution and persistence in wildlife and humans (half-life estimate 4-9 years)

Toxicity in Laboratory Animals: PFOA

- Hepatotoxic – hypertrophy, cytoplasmic lipid vacuoles, acidophilic degeneration/ necrosis
- Immunotoxic – Suppression, thymus and spleen atrophy
- Endocrine – elevated E_2, lowered $T_{4,}$ altered lipid metabolism
- Carcinogenic – in rat liver, pancreas, testes (Leydig cells)

Developmental Studies: PFOA

PFOA exposure produces developmental toxicity

- Dose-related pre- and postnatal lethality
- Dose-related postnatal growth deficits
- Developmental delay (delayed eye opening)
- Reproductive toxicity (delayed sexual maturation)
- Endocrine effects (Thyroid hormone imbalance)
- Mammary gland development

Cross-Foster Study:

- Does prenatal or postnatal exposure play more of a role in the effects of PFOA on the pup?
- Is *in utero* exposure alone sufficient?
- Is lactational exposure alone sufficient?
- Are both in utero and lactational exposure required?

Cross Foster groups:

Control:

- Control Pups + Control Dams = Control

Lactational exposure only:

- Control pups nursed to Dams dosed with 3 mg = 3L
- Control pups nursed to Dams dosed with 5 mg = 5L

In Utero exposure only:

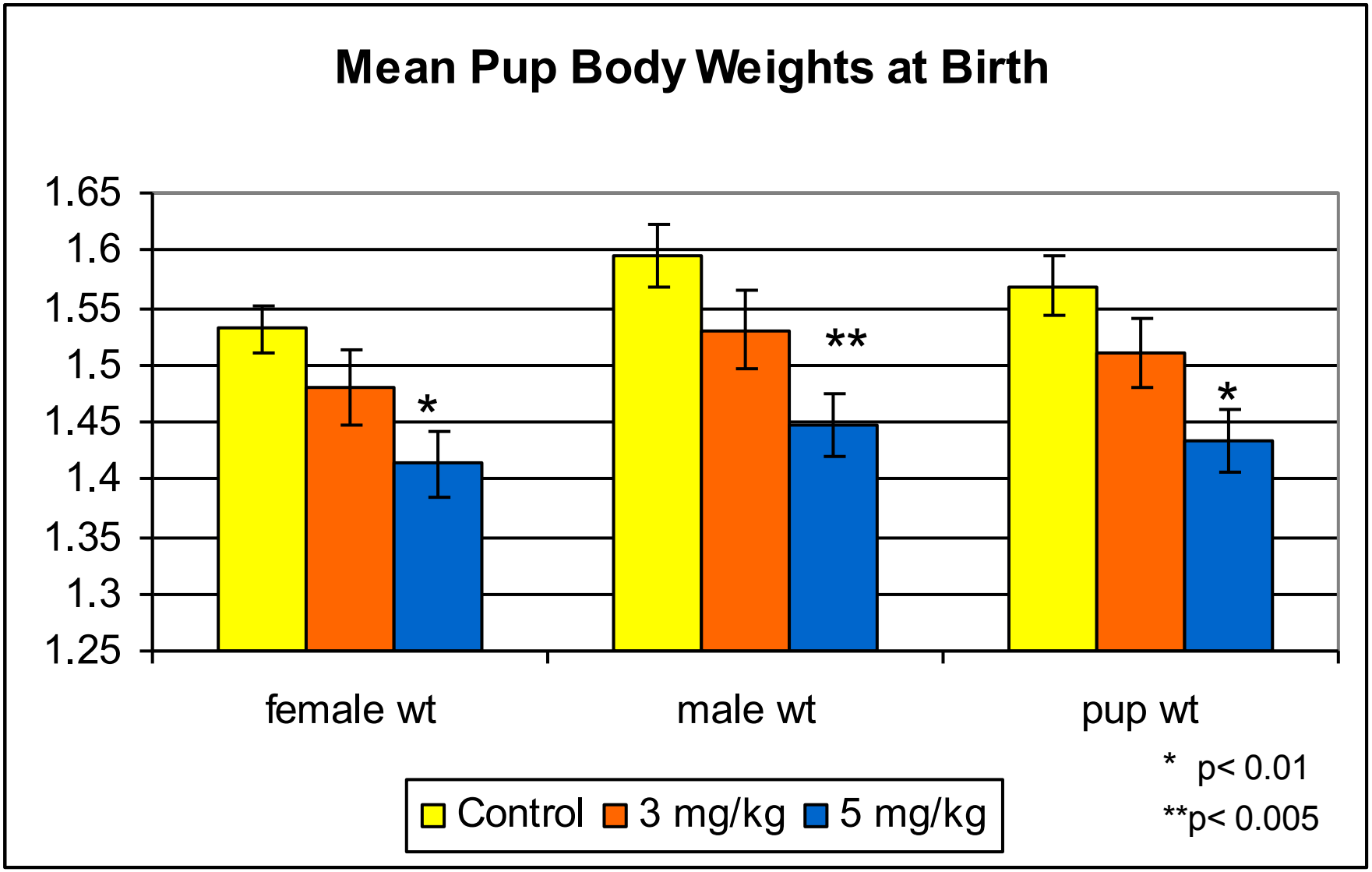
- Pups exposed to 3 mg in utero + Control dam = 3U
- Pups exposed to 5 mg in utero + Control dam = 5U

Both In utero and Lactational exposure:

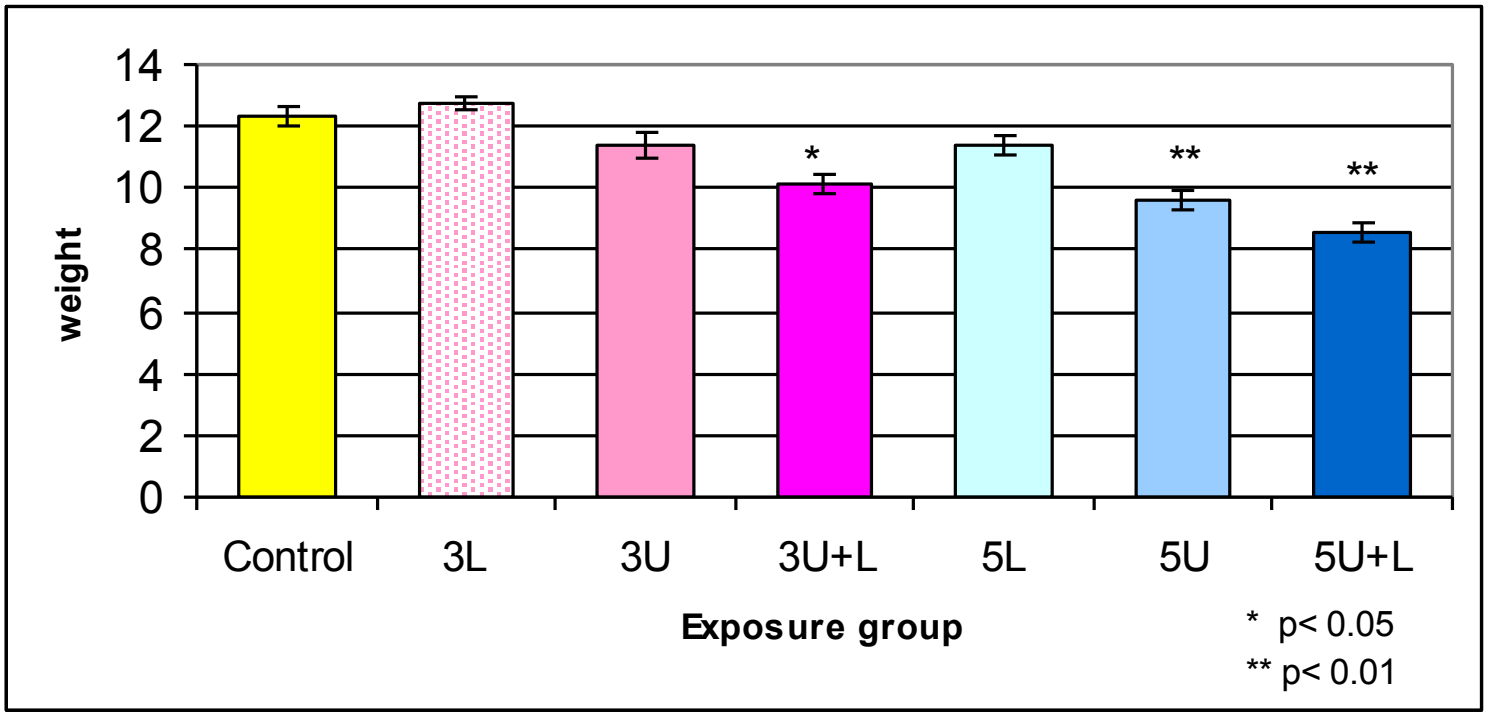
- Pups 3 mg/kg in utero + Dam 3 mg/kg = 3U+L
- Pups 5 mg/kg in utero + Dam 5 mg/kg = 5U+L

Cross Foster Study Outcomes:

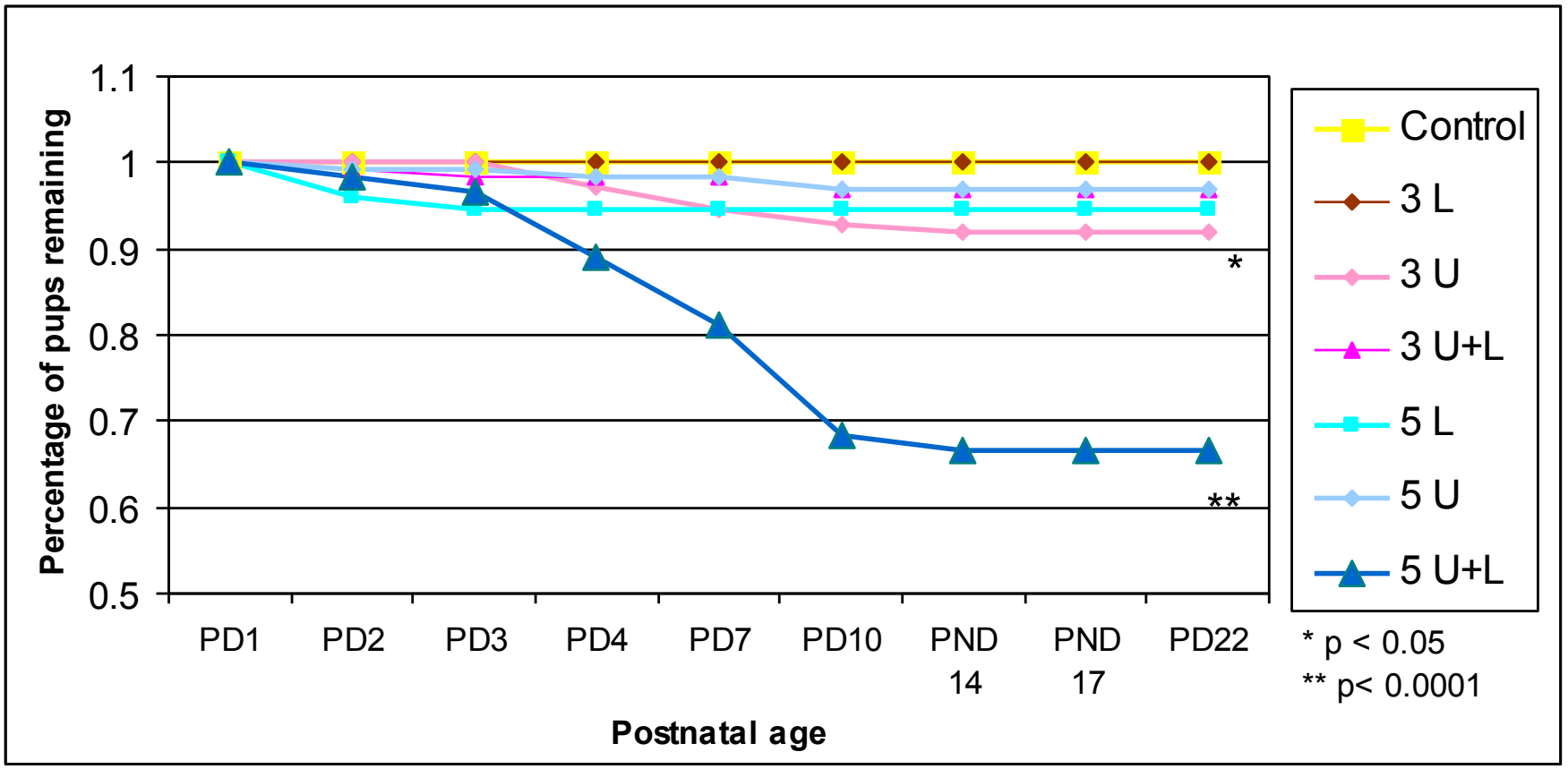
- Reduced body weights of offspring at birth and whole litters died *in utero* at 5 mg/kg PFOA
- Reduced survival of offspring throughout the first two weeks of life : 5 U+L
- Reduced pup weight gain and delayed eye opening and hair growth : 3 U+L, 5 U+L and 5 U
- Liver weight/body weight ratio increased in dams and offspring in all groups
- Post-weaning body weights remain lower in females to PND 85 : 5U, 5U+L


Mean Pup Body Weights at Birth
1.65
1.6
1.55
1.5
1.45
1.4
1.35
1.3
1.25
*
**
*
female wt
male wt
pup wt
Control
3 mg/kg
5 mg/kg
* p< 0.01
**p< 0.005

Fostered Pup Weight Gain PND1-22

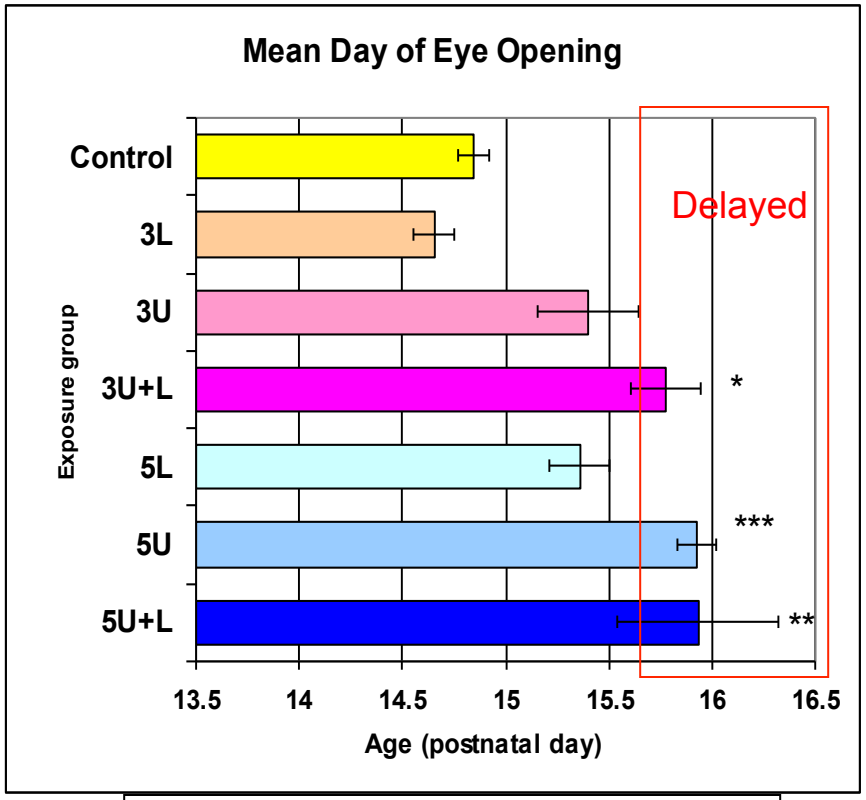


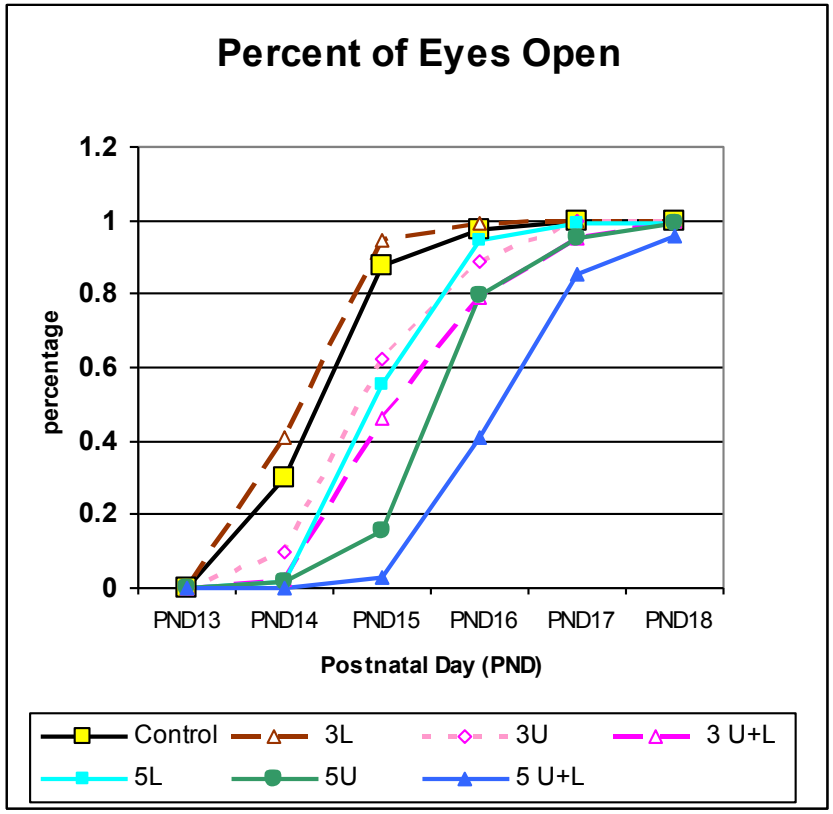
Survival of Fostered Litters



Eye Opening

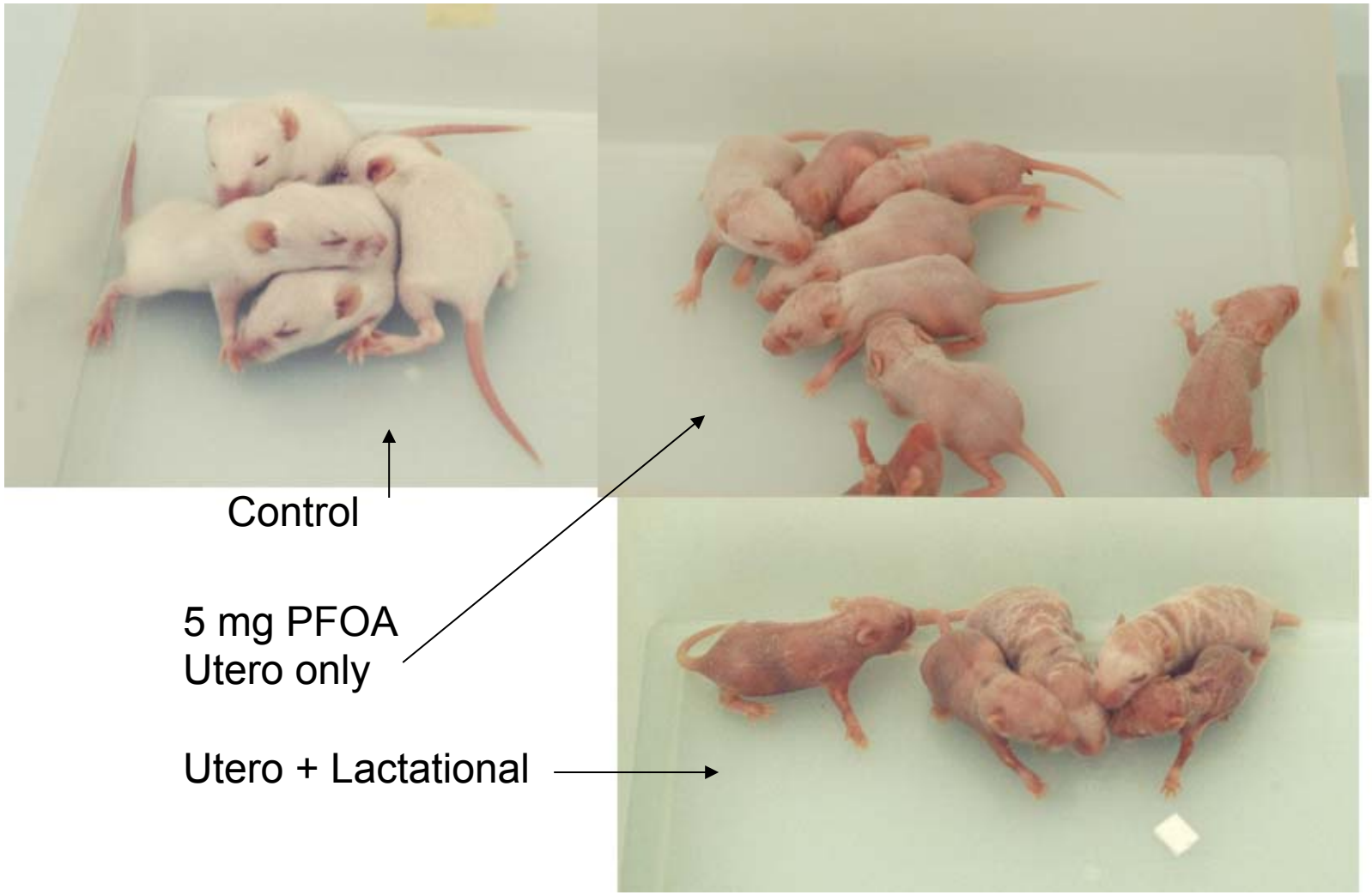
a landmark of development





* p< .05, ** p< 0.01, *** p<0.005

PND 11: Body Size & Hair Growth



Conclusions:

- *In utero* exposure is a major contributor to the effects of PFOA in the offspring
- *In utero* exposure alone can induce effects
- Lactational exposure may also contribute to effects on pup weight

PPARα, PPARβ, PPARγ

- PPARs are nuclear receptors that regulate lipid homeostasis, inflammation, adipogenesis, reproduction, wound healing
- PPAR isoforms have specific expression patterns during development in the embryo, placenta, and extra-embryonic membranes (amnion, yolk sac)
- Chemicals and drugs can activate PPAR pathways
- PPARα activation is considered to be a causal factor in PFOA-induced cancer in the rat
- Does PPARα mediate the PFAA-induced developmental toxicity?

PFAA-induced developmental toxicity & PPARα

In Vivo Studies:

- PPARα KO mice: Evaluate whether PFOA, PFNA, and PFOS have a PPARα-dependent mode-of-action for developmental toxicity

In Vitro Studies:

- Transfected cells to examine the potential for PFAA compounds to activate PPARα

Gene expression: QPCR and gene arrays:

- Characterize and compare gene expression profiles in response to PFOA or PFOS

Dose-response PFOA study in KO mice:

WT (129S1/SvImJ) and PPARα KO mice

- Mate overnight (plug+ = GD0)
- Dose by gavage GD1-17
- PFOA solution prepared daily in water
- 0, 0.1, 0.3, 0.6, 1, 3, 5, 10, or 20 mg/kg/day

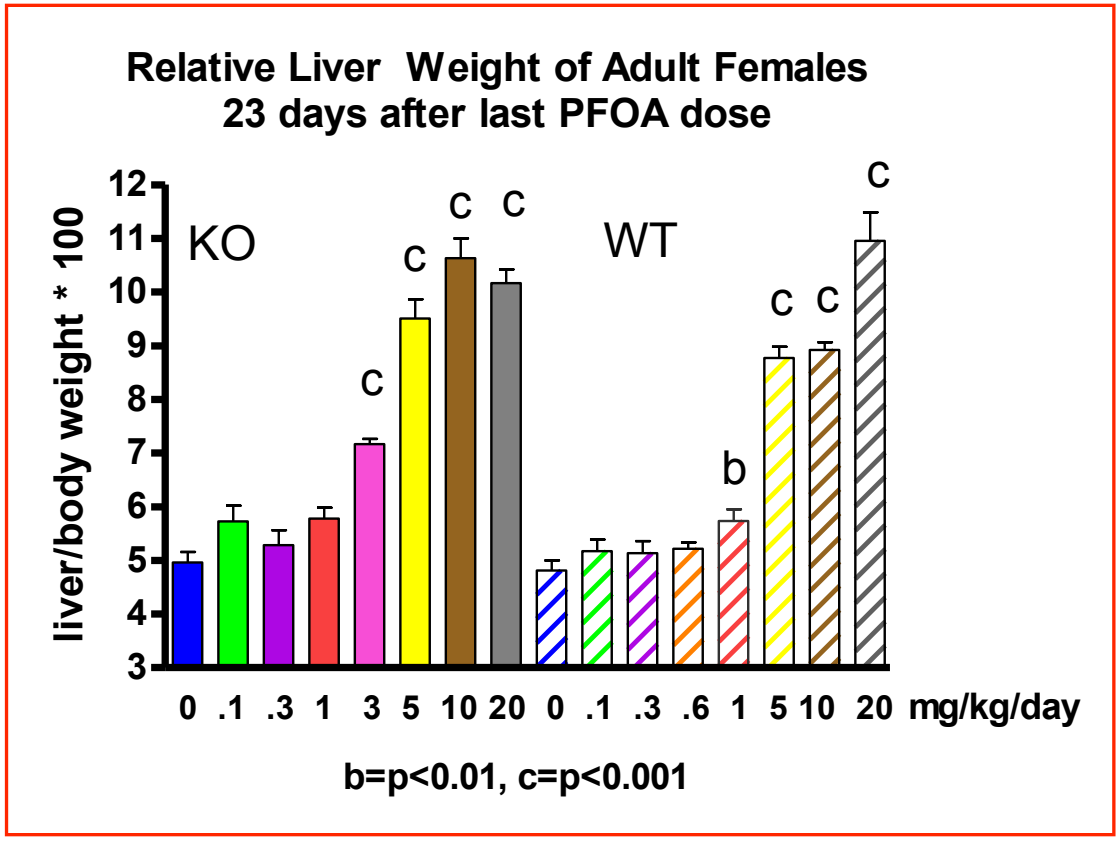
RESULTS:

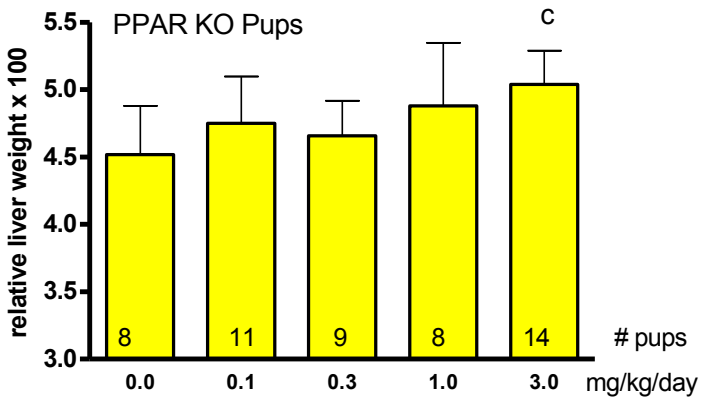
PFOA did not affect KO or WT:

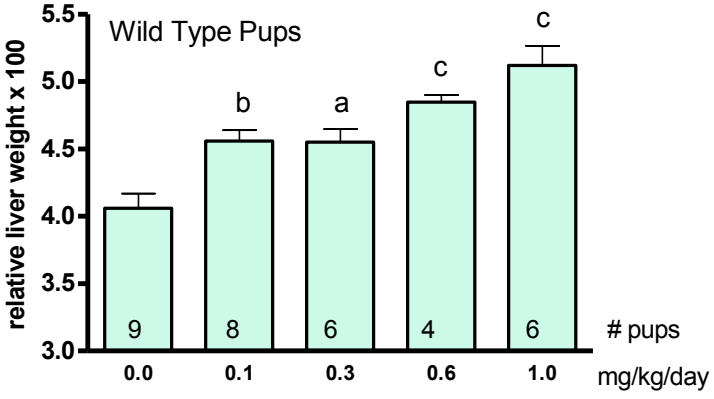
- Maternal weight gain
- # implanted embryos per dam
- Total # pups (live + dead) at birth
- Male or Female pup birth weights

In both KO and WT:

- PFOA increased early full litter resorption in both KO or WT (5 mg/kg/day or higher)
- Increased relative liver weight of adults and pups (WT pups at all doses; KO pups only slightly at 3 mg/kg)


Relative Liver Weight of Adult Females
23 days after last PFOA dose
liver/body weight * 100
KO
WT
c
b
0 .1 .3 1 3 5 10 20 0 .1 .3 .6 1 5 10 20 mg/kg/day
b=p<0.01, c=p<0.001

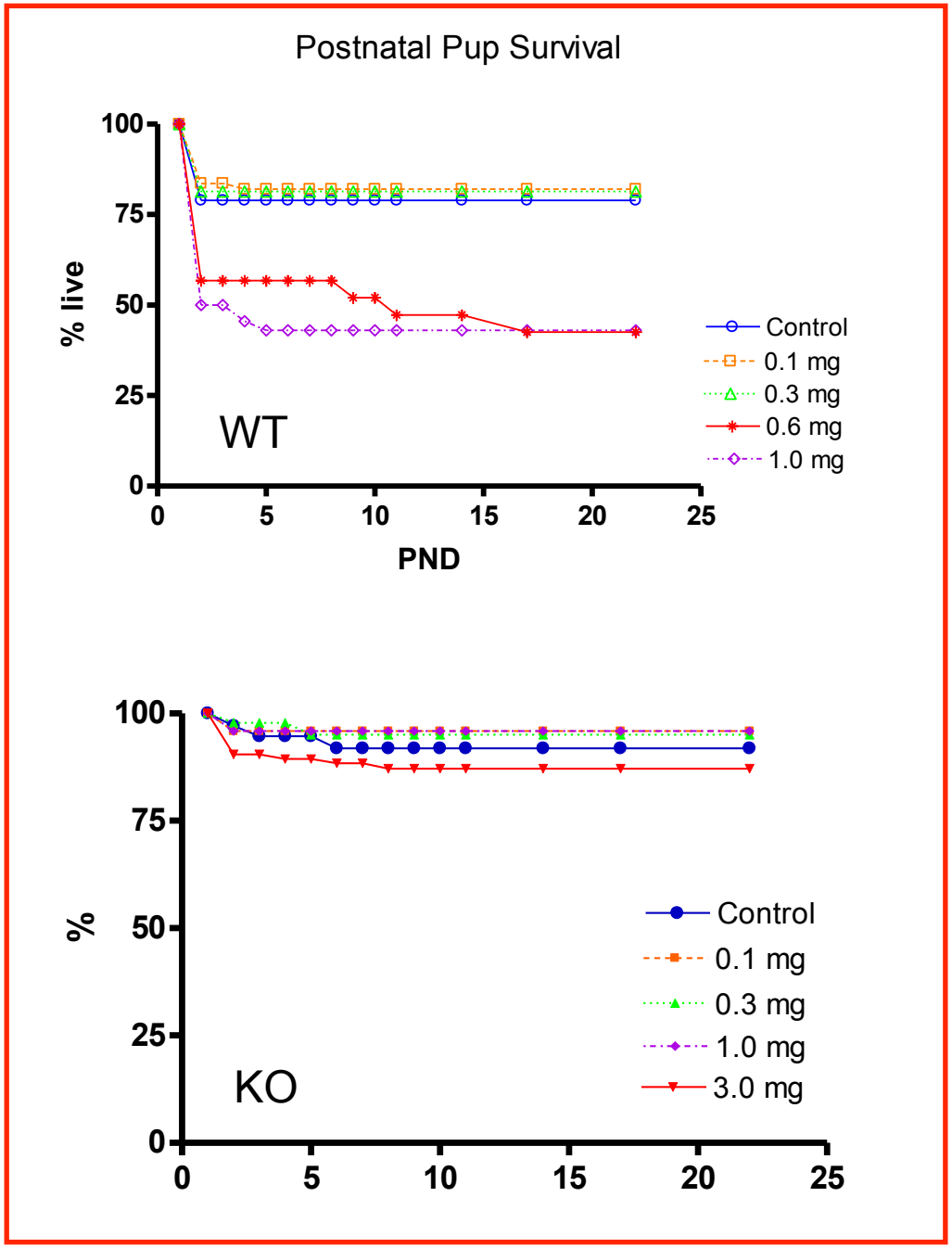

PPAR KO Pups
relative liver weight x 100
8 11 9 8 14
0.0 0.1 0.3 1.0 3.0
pups
mg/kg/day


Wild Type Pups
relative liver weight x 100
9 8 6 4 6
0.0 0.1 0.3 0.6 1.0
pups
mg/kg/day

RESULTS:

ONLY in WT did PFOA significantly

- Decrease pup survival PND1-22
- Decrease pup weight gain PND1-22
- Developmental delay (delayed eye opening)



Survival decreased in WT pups exposed to 0.6 or 1 mg PFOA, $p<0.001$.

No significant effect of PFOA on postnatal survival of PPAR KO pups.

The mean day of eye opening was delayed in WT by ~1 day ($p<0.05$).

Mean Day of Eye Opening					
Wild Type			PPAR KO		
Dose	n	Mean	Dose	n	Mean
0	**9**	**13.8±0.3**	**0**	**8**	**14.1±0.2**
0.1	**8**	**13.5±0.2**	**0.1**	**10**	**14.2±0.3**
0.3	**6**	**13.5±0.2**	**0.3**	**9**	**13.7±0.1**
0.6	**5**	**14.0±0.2**	**1**	**8**	**14.0±0.2**
1	**6**	**14.6±0.3***	**3**	**14**	**14.3±0.2**

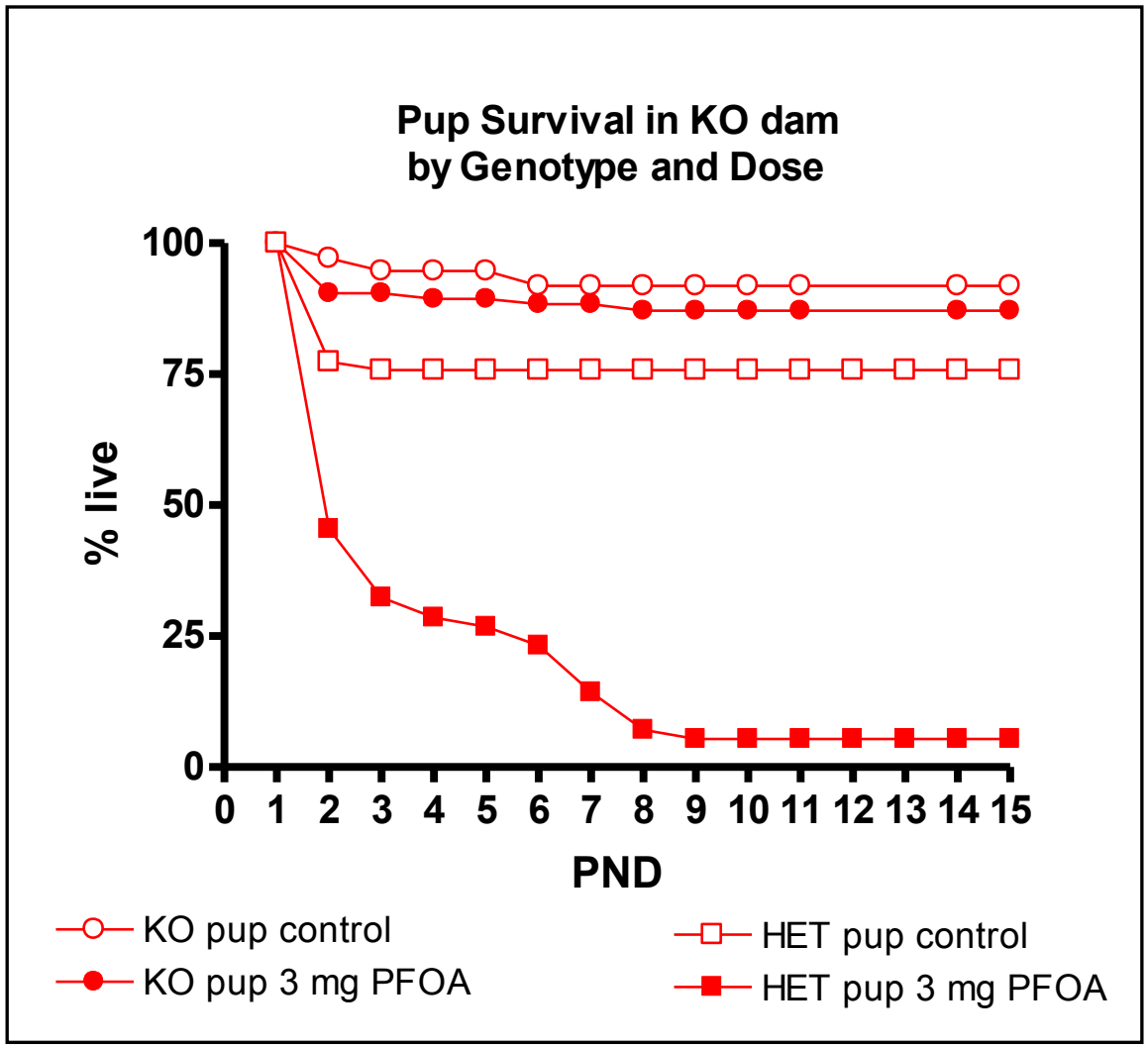
Serum levels and developmental toxicity of PFOA

		PND22 Serum level (ng/ml)	Relative liver weight increased	Survival PND1-22 decreased	Eye opening delayed	PND1-22 weight gain decreased
WT pups	1 mg/kg	9,860	√	√	√	√
KO pups	1 mg/kg	7,730	no	no	no	no
	3 mg/kg	10,600	slight	no	no	no

PFOA effects on postnatal pup survival:

WT pups die but KO pups live

- Is a potential difference in WT and KO genetic background a factor in survival of the KO pups?
- Are maternal factors involved?
  - effect of PFOA on WT dams contributing to pup mortality?
- Test these possibilities by exposing Heterozygous pups in WT and KO dams
  - WT female x KO male= all HET pups
  - KO female x WT male=all HET pups



Effects of PFOA on postnatal survival depend on expression of PPARα in the pup. Expression of even one copy of the PPARα gene results in decreased survival.

Dose-response PFNA study in KO mice:

WT (129S1/SvImJ) and PPARα KO mice

- Mate overnight (plug+ = GD0)
- Dose by gavage GD1-17
- PFNA solution prepared daily in water
- 0, 0.83, 1.1, 1.5, or 2.0 mg/kg/day

RESULTS:

PFNA did not affect KO or WT:

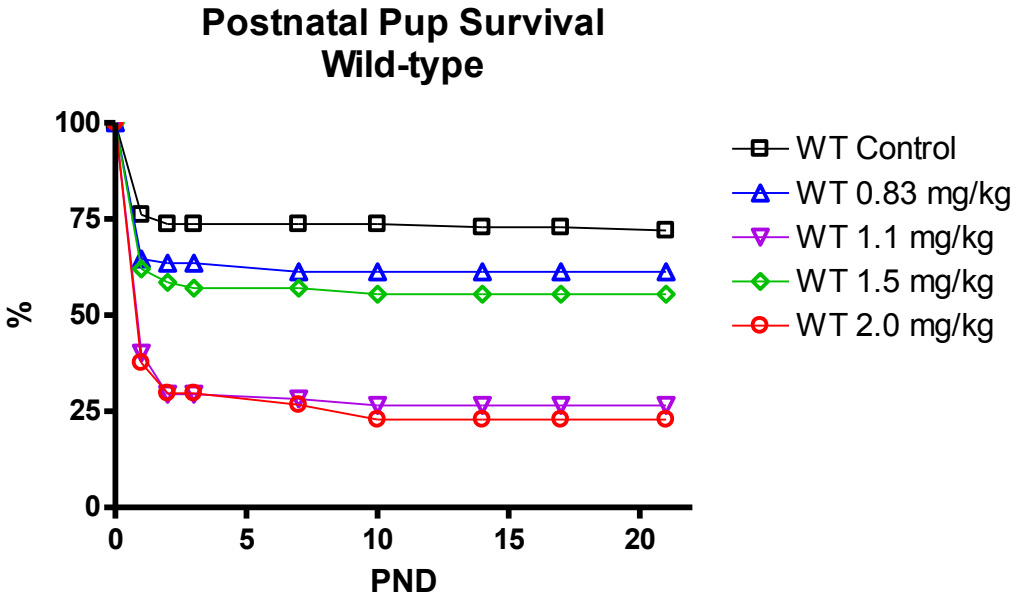
- Maternal weight gain
- # implanted embryos per dam
- Male or Female pup birth weights

WT only:

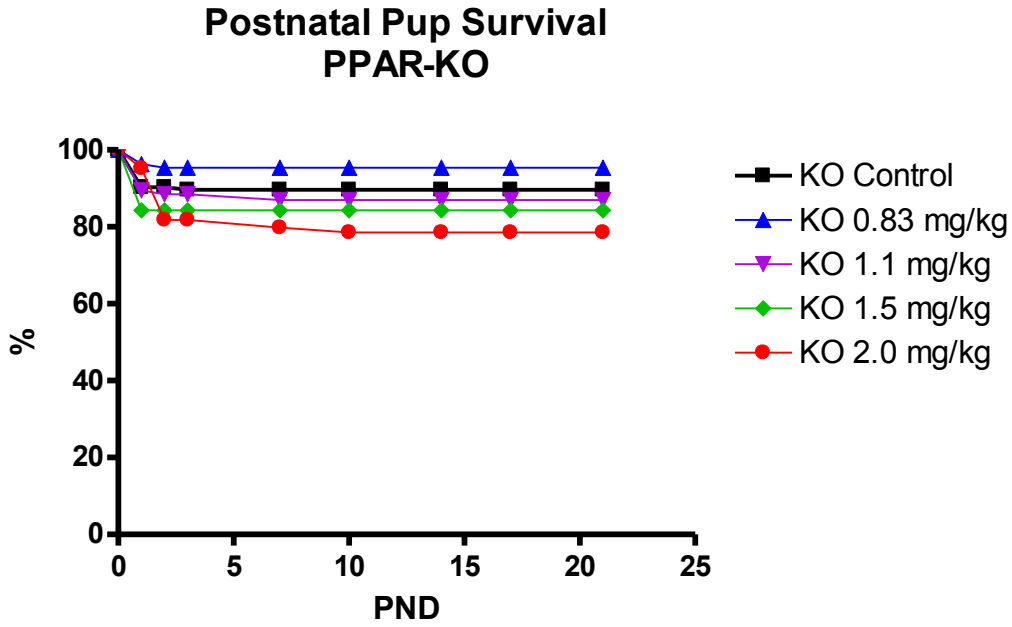
- PFNA decreased # live per litter at 1.1 and 2.0 mg/kg
- FLR increased at 2.0 mg/kg

WT and KO:

- Relative liver weight increased in WT adult and pups (all doses)
- KO pups only slightly at 2 mg/kg



Survival decreased in WT pups exposed to 1.1 or 2 mg PFNA



No significant effect of PFNA on postnatal survival of PPAR KO pups.

Serum levels and developmental toxicity of PFNA

		PND22 Serum level (ng/ml)	Relative liver weight increased	Survival PND1-22 decreased	Eye opening delayed	PND1-22 weight gain decreased
WT pups	1.1 mg/kg	15,700	√	√	no	no
	2.0 mg/kg	25,300	√	√	√	√
KO pups	1.1 mg/kg	19,400	no	no	no	no
	2.0 mg/kg	38,400	slight	no	no	no

Dose-response PFOS study in KO mice:

- WT and PPARα KO mice

  Mate overnight (plug+ = GD0)

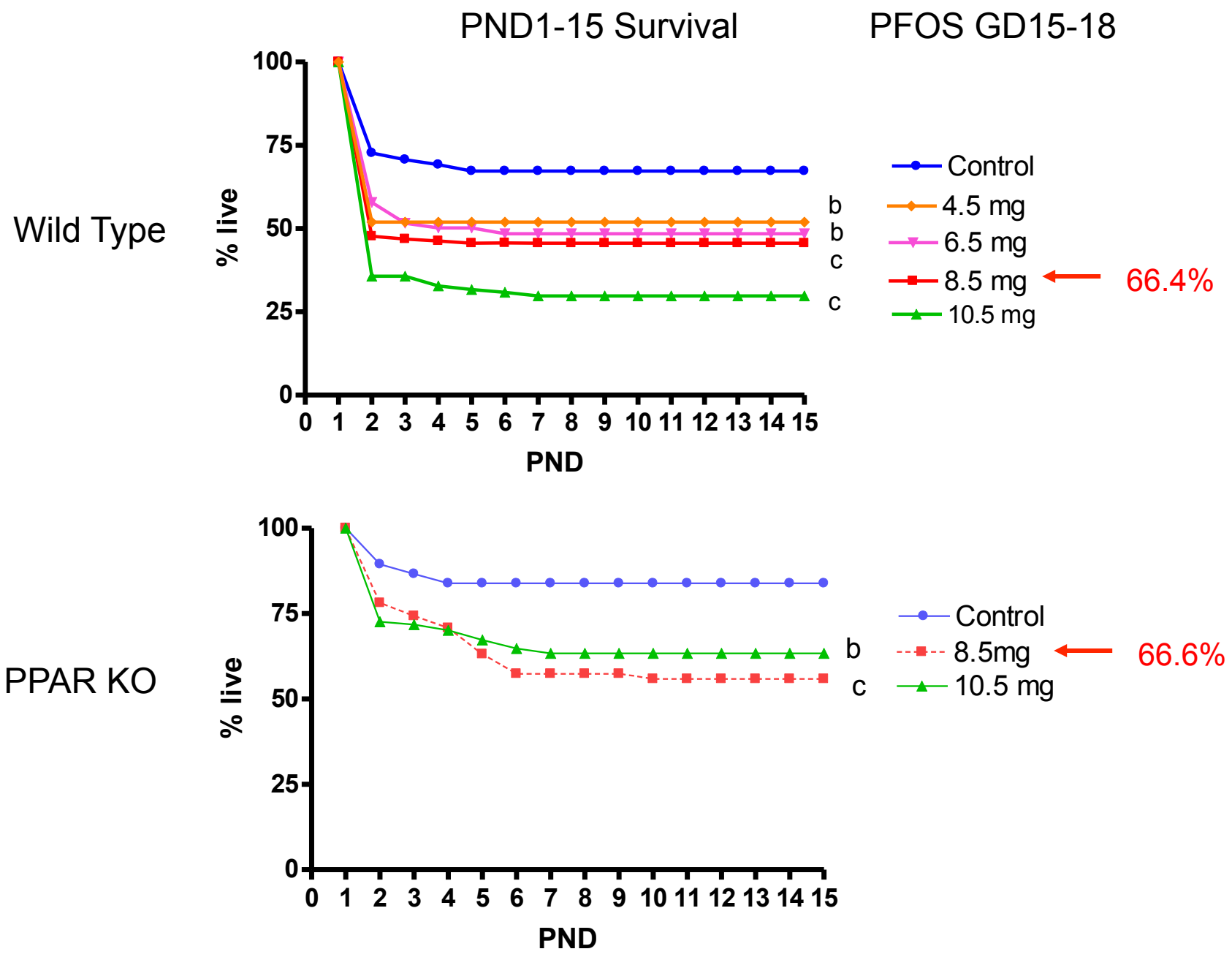
  - Dose by gavage GD15-18
  - PFOS solution prepared daily in 0.5% Tween-20
  - WT : 0, 4.5, 6.5, 8.5, or 10.5 mg/kg/day
  - KO : 0, 8.5, or 10.5 mg/kg/day
- Evaluate pup survival, weight gain, eye opening from PND1-15

RESULTS:

In both WT and PPAR KO

PFOS did not affect:

- Maternal weight gain
- # implanted embryos per dam
- % litter loss from implantation to birth
- Total # pups (live + dead) at birth
- Male or Female pup birth weights
- Pup body weight or weight gain PND1-15


PND1-15 Survival
PFOS GD15-18
Wild Type
% live
PND
b
b
c
c
Control
4.5 mg
6.5 mg
8.5 mg
10.5 mg
66.4%
PPAR KO
% live
PND
b
c
Control
8.5mg
10.5 mg
66.6%

Serum levels and developmental toxicity of PFOS

		PND15 Serum level (ng/ml)	Relative liver weight increased	Survival PND1-15 decreased	Eye opening delayed	PND1-15 weight gain decreased
WT pups	8.5 mg/kg	40,700	no	√	√ PND13	no
	10.5 mg/kg	41,200	√	√		no
KO pups	8.5 mg/kg	42,800	no	√		no
	10.5mg/kg	52,400	√	√	√ PND14	no

PFOS Developmental Toxicity:

PPARα independent mode of action

- WT and KO neonates die

PFOS does not depend on expression of PPARα to produce neonatal lethality, some other mode of action occurs

PFOS studies in rat (Grasty et al 2005) suggest effects on lung maturation or function

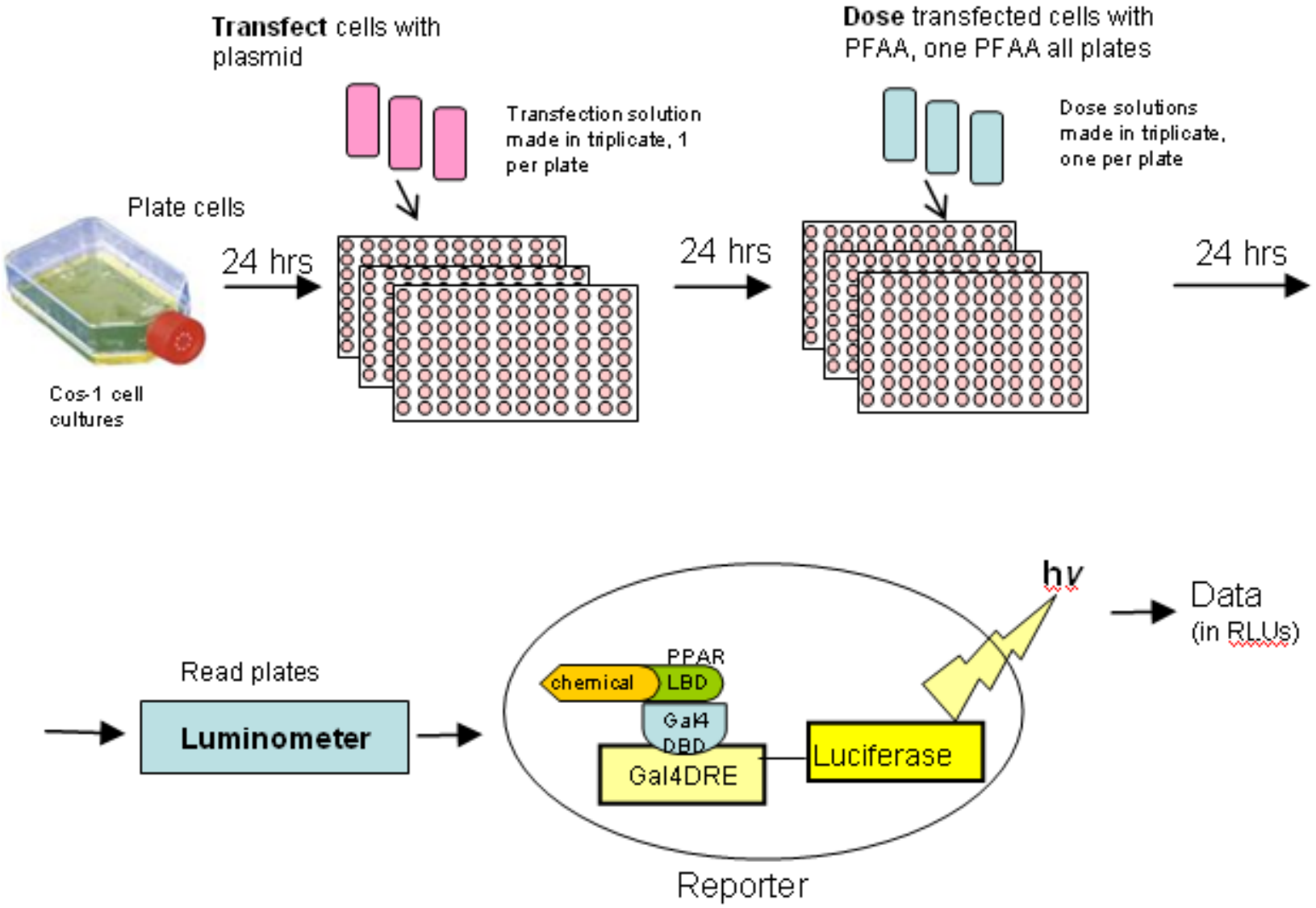
- Newborn rats appeared to have difficulty breathing
- Lungs appeared small or underinflated
- Histologically the lungs appeared immature
- Initial impressions were of effects on lung maturation

PPARα Mode-of-Action for other PFAAs?

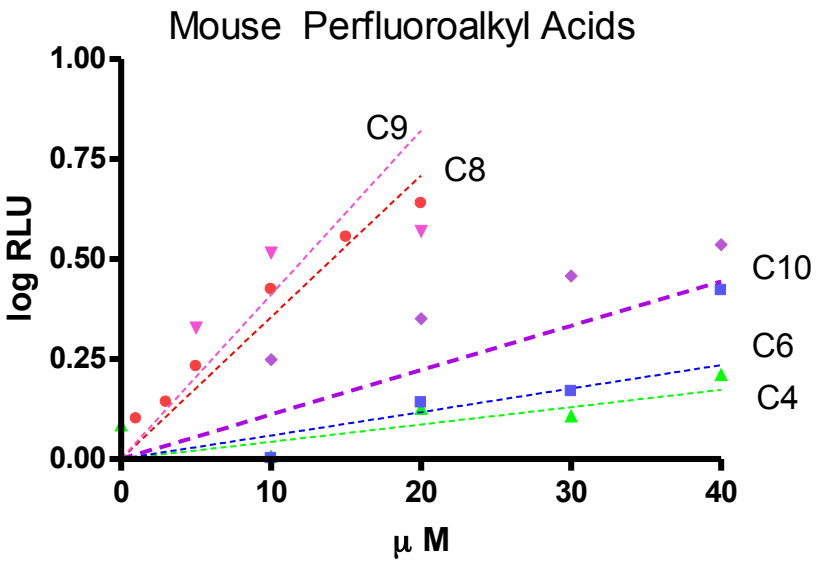
- Test potential for other PFAAs to activate PPARα
  - In Vitro Assay using transiently transfected Cos-1 cells
  - Plasmid containing the mouse or human PPARα ligand binding domain (LBD)
  - Activation evaluated with a Luciferase reporter
- Compare:
  - PFAAs of various carbon chain lengths
  - Perfluorocarboxylates vs Sulfonates
  - Activity of PFAAs on mouse vs human PPARα
  - Evaluate mixture behaviors (additivity?)

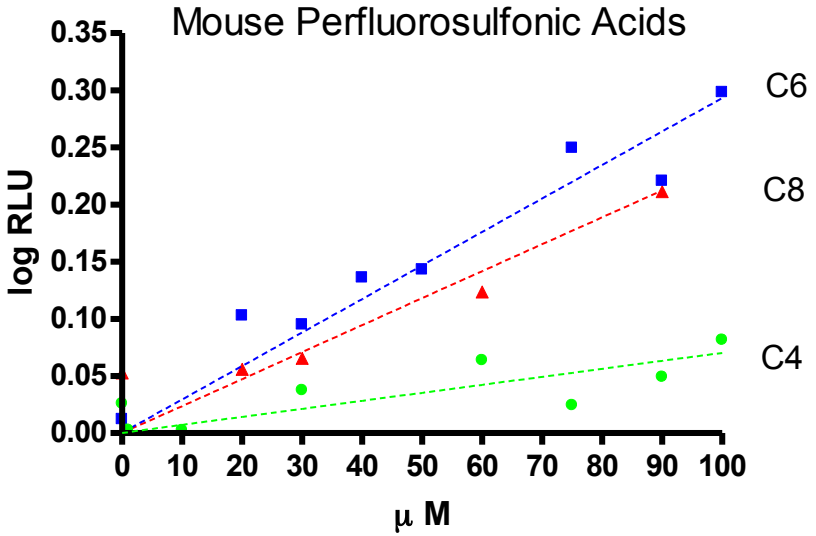
Mouse and Human PPAR plasmids provided by Jeff Peters and Jack Vanden Heuvel, Penn State University, PA

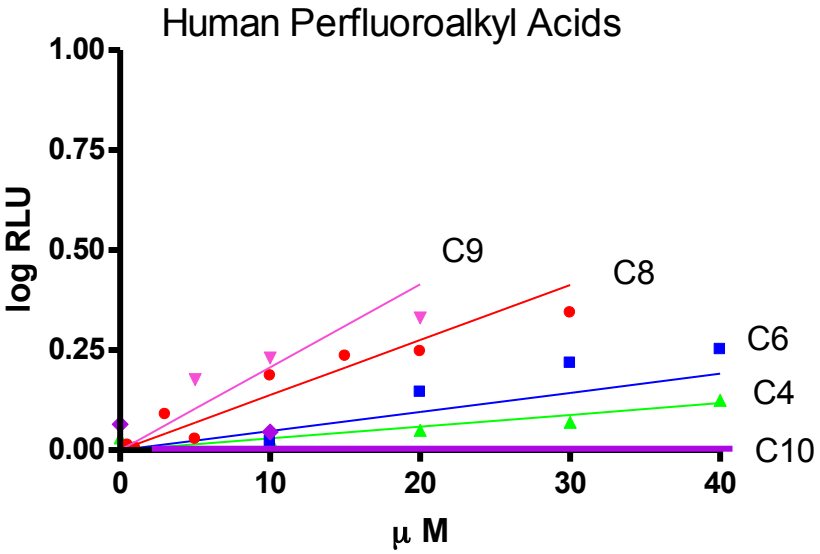
Transient Transfection Assay

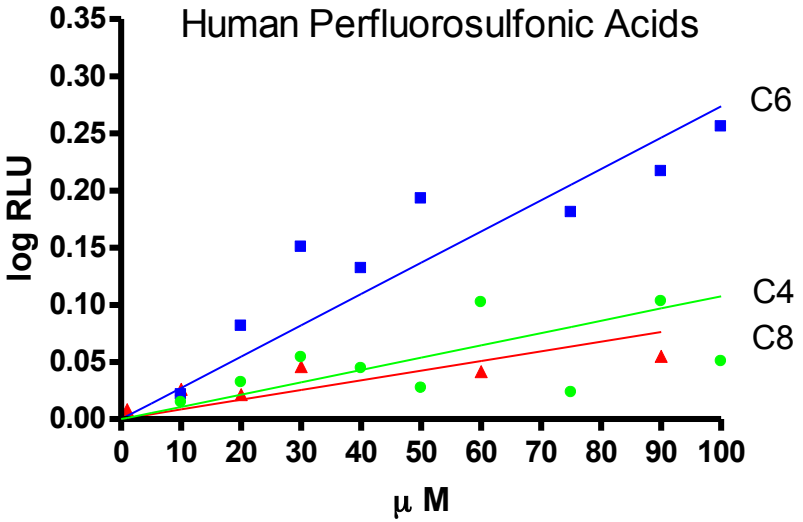


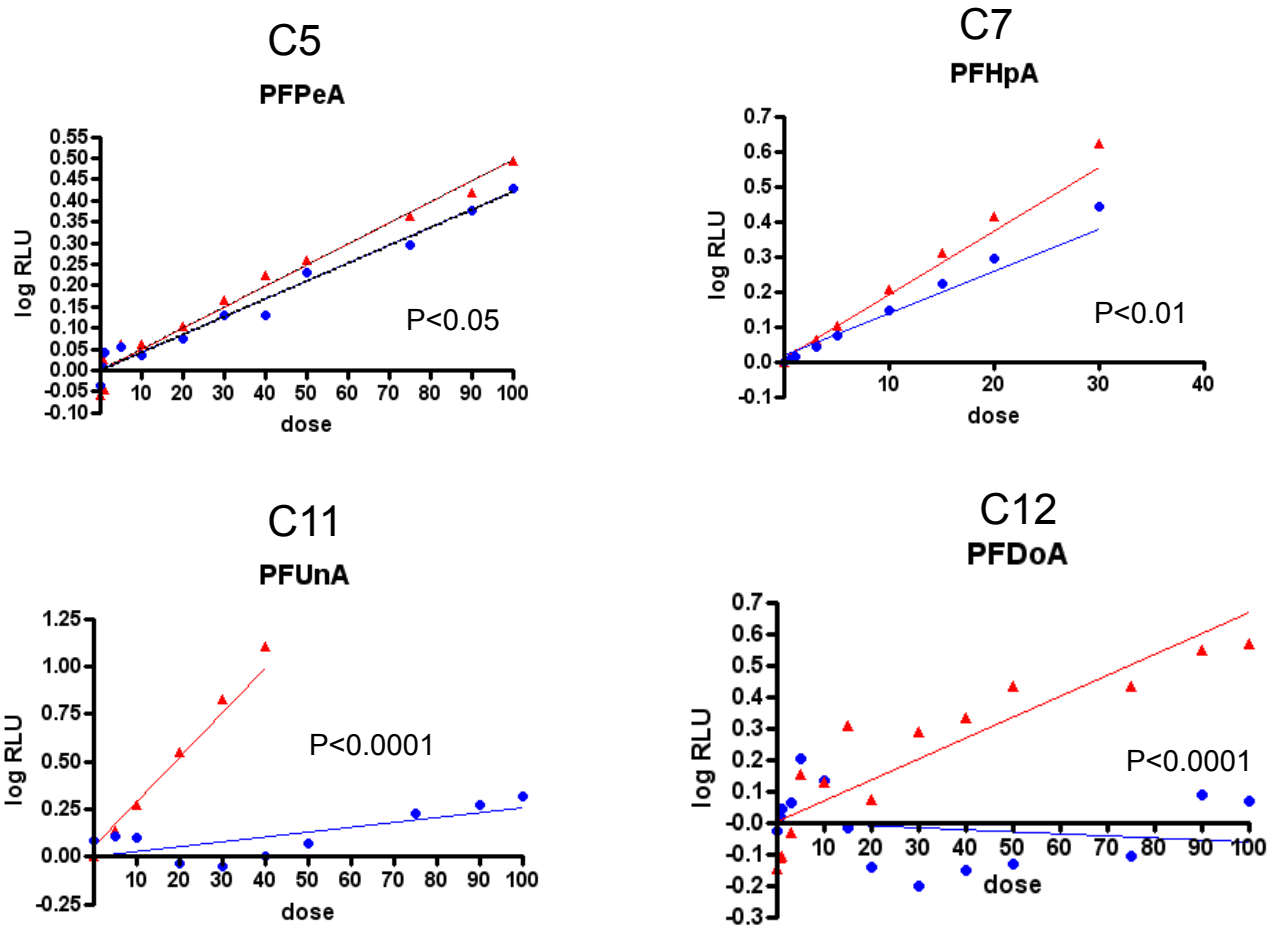
Test compounds:	Carbon chain length
Perfluorobutanoic acid (PFBA)	4
Perfluoropentanoic acid (PFPeA)	5
Perfluorohexanoic acid (PFHxA)	6
Perfluoroheptanoic acid (PFHpA)	7
Perfluorooctanoic acid (PFOA)	8
Perfluorononanoic acid (PFNA)	9
Perfluorodecanoic acid (PFDA)	10
Perfluoroundecanoic acid (PFuNA)	11
Perfluorododecanoic Acid (PFDoA)	12
Perfluorobutane sulfonate (PFBS)	4
Perfluorohexane sulfonate (PFHxS)	6
Perfluorooctane sulfonate (PFOS)	8


Mouse Perfluoroalkyl Acids
log RLU
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
μ M
C9
C8
C10
C6
C4


Mouse Perfluorosulfonic Acids
log RLU
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
90
100
μ M
C6
C8
C4


Human Perfluoroalkyl Acids
log RLU
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
μ M
C9
C8
C6
C4
C10


Human Perfluorosulfonic Acids
log RLU
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
90
100
μ M
C6
C4
C8



Mouse and human responses were compared by regression analysis.
▲ mouse plasmid, ● human plasmid.
P values shown on plots are the level of significance of difference between the slopes of the regression lines for mouse vs. human.
NS, not significant.

PFAA Activities on PPARα in Transfected COS-1 Cells

	C_{20max} (μM)	
Compound	***Mouse***	***Human***
PFBA (C4)	51	75
PFPeA (C5)	45	52
PFHxA (C6)	38	47
PFHpA (C7)	11	15
PFOA (C8)	6-7	7-16
PFNA (C9)	5	11
PFDA (C10)	20	no activity
PFUnDA (C11)	8	86
PFDoDA (C12)	33	no activity
PFBS (C4)	317	206
PFHxS (C6)	76	81
PFOS (C8)	94	262

Abbott, Wolf, et al

Summary of In Vitro PPARα Assay

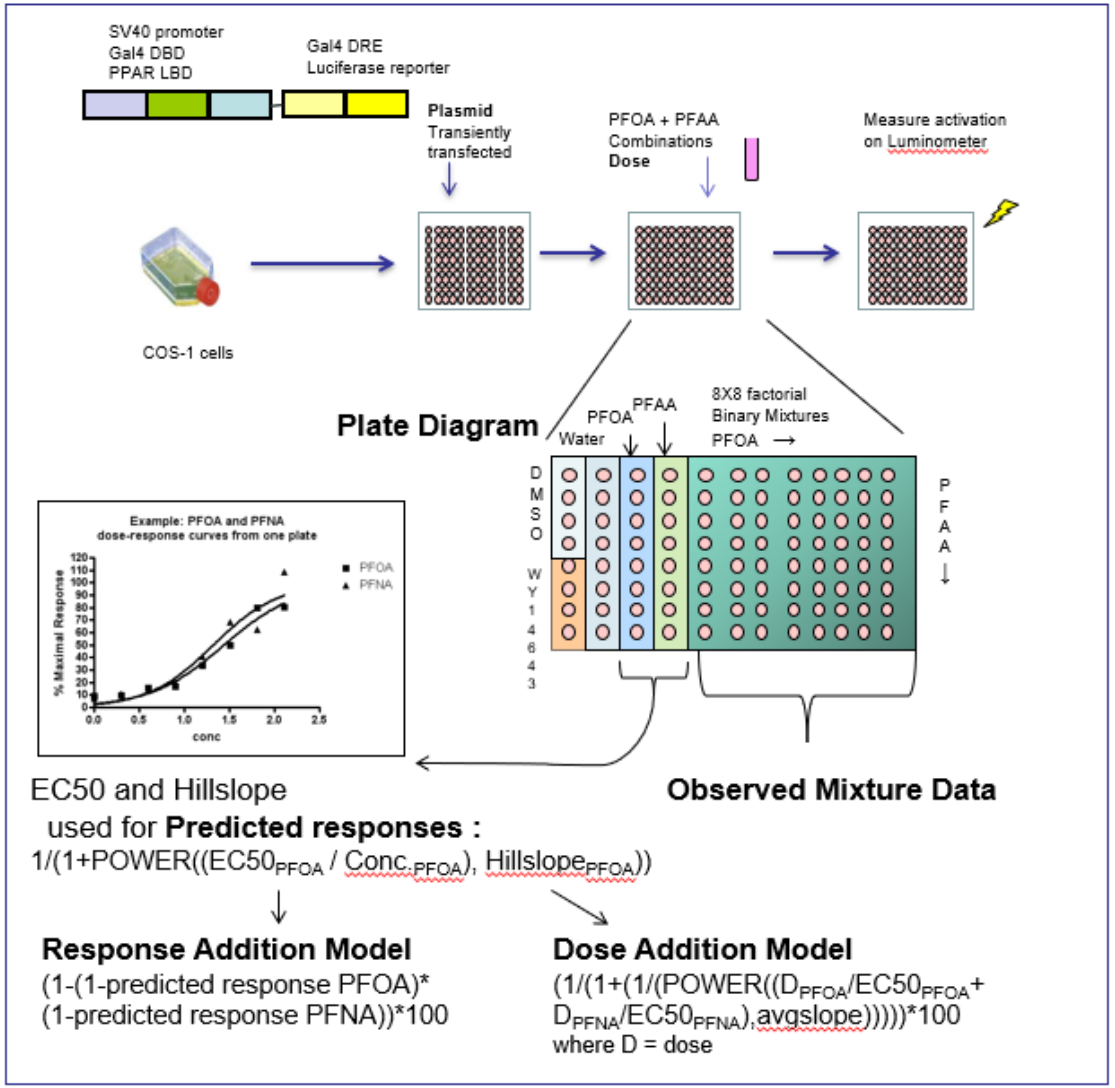
- *In vitro* assay shows that many of the PFAAs have the potential to act via a PPARα mode-of-action
- Perfluorocarboxylates are more active than sulfonates
- Activity increases with increasing chain length
- Mouse PPARα is more responsive than human

PFAA Mixtures

- Activation of mouse PPARα in transfected COS-1 cells
- Binary combinations of PFOA and PFHxA, PFNA, PFHxS or PFOS in μM range were evaluated
- Interactions between PFOA and the 4 PFAAs examined were largely additive
- Results are comparable with those using a fixed-ratio mixture of 4 PFAAs (PFOS, PFOA, PFHxS and PFNA) based on NHANES data

PFAA test chemicals: **Concentration ranges** (at two-fold increments)

perfluorooctanoic acid (PFOA)	1 – 128 µM
perfluorononanoic acid (PFNA)	1 – 128 µM
perfluorooctane sulfonate (PFOS)	4 – 384 µM
perfluorohexanoic acid (PFHxA)	8 – 1024 µM
perfluorohexane sulfonate (PFHxS)	8 – 2048 µM

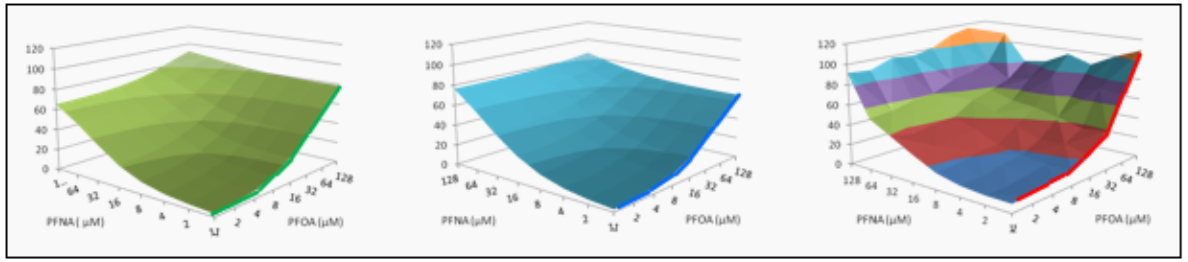
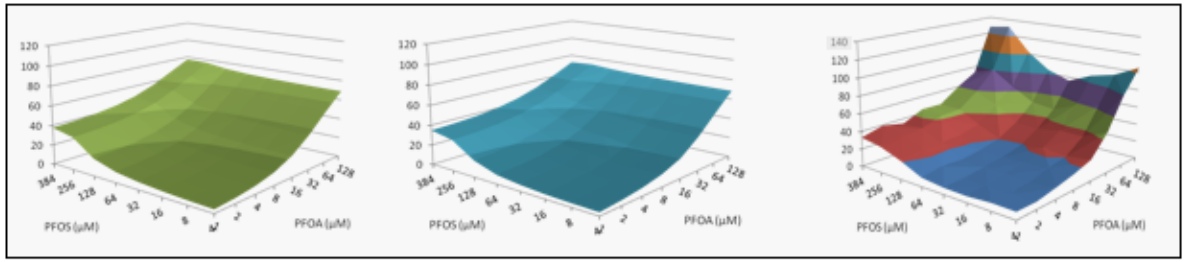
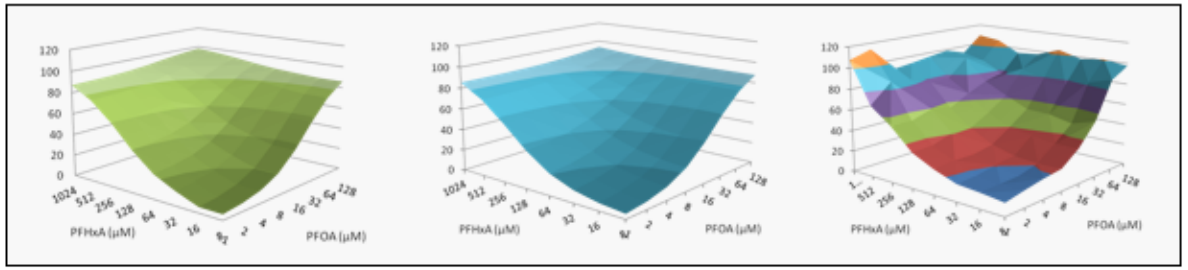
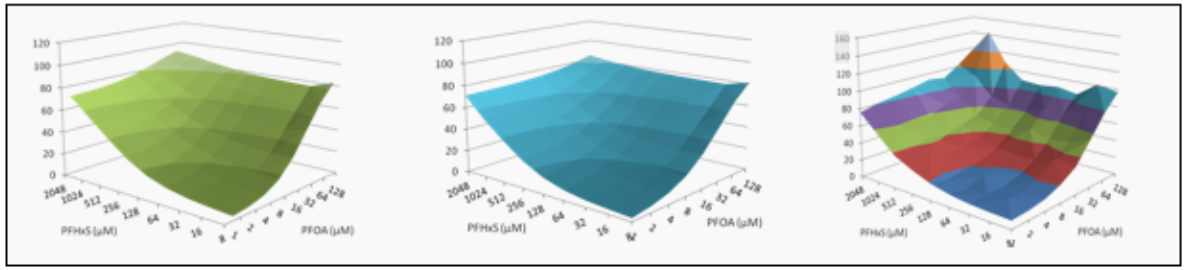


Surface Plots of PPARα Activation by Binary Mixtures of PFAAs
Modeled and Observed Results

Graphs of Response Addition, Dose Addition and Observed data below represent means of all plates per PFOA + PFAA combination. Dose response curves from individual PFAAs from each plate generated EC50s and Hillslopes used to derive models for the mixtures for that plate. Means of all plates were used to make surface plots.

Response Addition Model
(1-(1-predicted response PFOA)*
(1-predicted response PFNA))*100

Dose Addition Model
(1/(1+(1/(POWER((D_{PFOA}/$EC50_{PFOA}$+
D_{PFNA}/$EC50_{PFNA}$),avgslope)))))*100

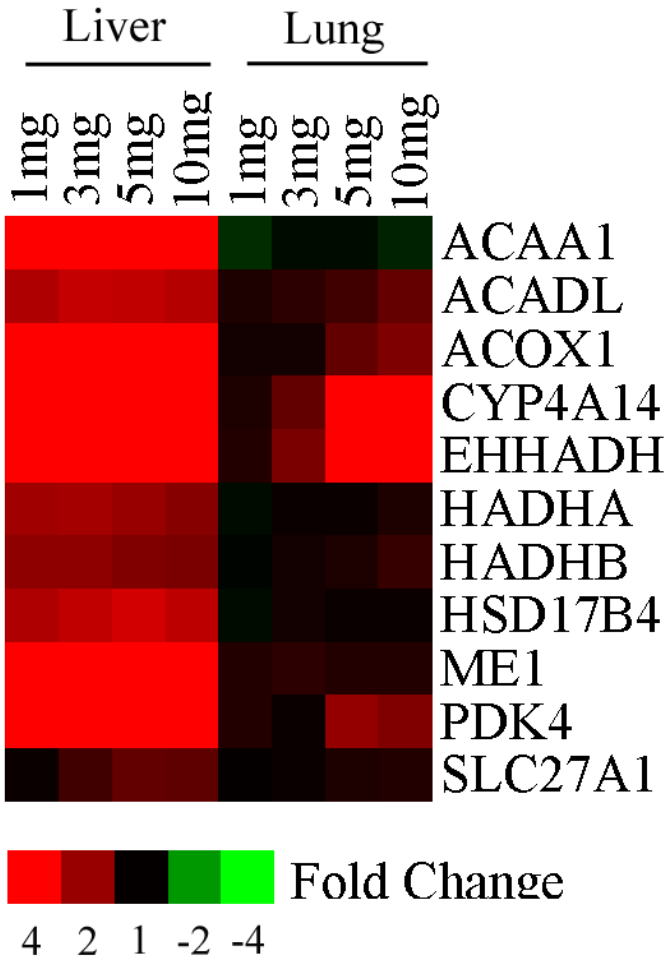
	Response Addition Model	Dose Addition Model	Observed data
PFNA			
PFOS			
PFHxA			
PFHxS			

Gene Expression in Fetal Tissues (Gene Array)

- Timed-pregnant CD-1 mice
- 0, 1, 3, 5, 10 mg/kg PFOA
- 0, 5, 10 mg/kg PFOS
- Dosed from GD 1-17 by oral gavage
- GD17 fetal liver and lung total RNA prepared
- Five biological replicates per group (pool litter)
- Gene profiling using Affymetrix 430_2 microarrays

Effect of PFOA and PFOS on PPARα marker genes in the mouse fetus

PFOA



PFOS

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Gene signatures altered by PFOA in the fetal mouse liver

	PFOA	PFOS
▪ Lipid metabolism and transport	+++	+++
▪ Peroxisome biogenesis	+++	+++
▪ Xenobiotic metabolism	++	+
▪ Acute phase response	++	
▪ Proteasome activation	++	
▪ Cholesterol biosynthesis	++	
▪ Phospholipid metabolism	++	+
▪ Bile Acid Biosynthesis	++	+
▪ Glucose metabolism	++	+

Gene expression in pre- and post-natal liver & heart (qPCR)

CD-1 Mice Dose GD1-17 PFOA 5 mg/kg/day

Collect fetal & postnatal tissues GD14, GD17, PND1, 14, 21, 28, (42 & 63 for liver)

Mouse Liver & Heart: PFOA induction of genes

Liver: GD14, GD17, PND1, 14, 21, 28, 42, & 63
Heart: GD14, GD17, PND1, 14, 21, 28

PPARα regulated

• Acox1	peroxisomal	Fatty Acid β-oxidation
• Ehhadh	peroxisomal	Fatty Acid β-oxidation
• Pdk4	mitochondrial	Glucose metabolism
• Cyp4a14	microsomal	Fatty Acid oxidation
• Me1	cytosolic	Fatty Acid biosynthesis
• Acaa1	peroxisomal	Fatty Acid metabolism

CAR and PPARα regulated

• Cyp2b10	microsomal	Arachadonic Acid and xenobiotic metabolism

PXR regulated (liver)

• Cyp3a11	microsomal	lineoleic acid metabolism

PPARγ (heart)

• Pgc1a	PPARγ signaling	Fatty Acid oxidation
• Cpt1b	PPAR signaling	Fatty Acid metabolism

Final Slide! Take home message—

Animal models revealed
- species differences
- pharmacokinetic influences
- confirmed a role for PPAR

In vitro model of receptor activation
- comparing larger numbers of PFAAs
- comparing relative activity of PFAAs
- evaluating binary and complex mixture behaviors

Overall the animal and in vitro models promoted
- understanding the basis for a developmental response
- evaluation of risk to human health

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