DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 82925

Title: Health-relevant phenotypes in the offspring of mice given CAR activators prior to pregnancy

Authors: Karin Dietrich, Jan Baumgart, Leonid Eshkind, Lea Reuter, Ute Gödtel-Armbrust, Elke Butt, Michael Musheev, Federico Marini, Piyush More, Tanja Grosser, Christof Niehrs, Leszek Wojnowski, Marianne Mathäs

Affiliations:

Department of Pharmacology, University Medical Center Mainz, Mainz, Germany (K. D., L. R., U. G.-A., P. M., T. G. L.W., M. Ma.)

Translational Animal Research Center, University Medical Center, Johannes Gutenberg-Universität Mainz, Mainz, Germany (J. B.)

Transgenic Facility Mainz, Translational Animal Research Center, University Medical Center, Johannes Gutenberg University Mainz, Mainz, Germany (L. E.)

Institute of Experimental Biomedicine II, University Hospital Würzburg, Würzburg, Germany (E. B.)

Institute of Molecular Biology, Mainz, Germany (M. Mu, C. N.)

Institute of Medical Biostatistics, Epidemiology and Informatics, University Medical Center Mainz, Mainz, Germany (F. M.)

Division of Molecular Embryology, German Cancer Research Center (DKFZ-ZMBH Alliance), 69120 Heidelberg, Germany (C. N.)

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 82925

Running Title: Soft inheritance of hepatic induction

Corresponding author:

Dr. Marianne Mathäs

address: Obere Zahlbacher Straße 67, 55131 Mainz

phone: 0049 6131 179219

e-mail: mathaes@uni-mainz.de

Number of text pages: 14

Number of figures: 7

Number of references: 84

Number of words in the Abstract: 185

Number of words in the Introduction: 392

Number of words in the Discussion: 1362

Abbreviations:

CAR, constitutive androstane receptor (NR1I3)

F1, first filial generation

HDL, high-density lipoprotein

HFD, high fat diet

i.p., intraperitoneal

LDL, low-density lipoprotein

logP, logarithmic partition coefficient

pKa, logarithmic acid dissociation constant

PXR, pregnane x receptor (NR1I2)

s.c., subcutaneous

SD, standard diet

TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene

VLDL, very low-density lipoprotein

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The hepatic induction in response to drugs and environmental chemicals affects drug therapies and energy metabolism. We investigated whether the induction is transmitted to the offspring. We injected 3-day and 6-week-old F0 female mice with the activator of the nuclear receptor CAR (constitutive androstane receptor, NR1I3) TCPOBOP and mated them 1-6 weeks afterwards. We detected in the offspring long-lasting alterations of CAR-mediated drug disposition, energy metabolism, and lipid profile. The transmission to F1 was mediated by TCPOBOP transfer from the F0 adipose tissue via milk, as revealed by embryo transfer, cross-fostering experiments, and liquid chromatography-mass spectrometry analyses. The important environmental pollutant PCB153 activated CAR in the F1 generation similarly to TCPOBOP. Our findings indicate that chemicals accumulating and persisting in adipose tissue may exert liver-mediated, health-relevant effects on F1 offspring simply via physical transmission with milk. Such effects may occur even if treatment has been terminated far ahead of conception. This should be considered in assessing developmental toxicity and in the long-term follow-up of offspring of mothers exposed to both approved and investigational drugs, and to chemicals known or suspected of accumulation in adipose tissue.

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Activation of the nuclear receptors CAR and PXR by drugs and environmental pollutants induces the hepatic expression of genes involved in drug and energy metabolism (Prakash et al., 2015). This accelerates the disposition, and thereby reduces the efficacy of numerous drugs such as oral anti-retrovirals, contraceptives, and immunosuppressants. Alterations in the metabolism of endobiotics such as steroids, bile acids, bilirubin, retinoids, carbohydrates, and lipids (Moreau et al., 2008) affect, among others, the risk of diabetes, obesity (Yang and Wang, 2014), and fatty liver disease (Yamazaki et al., 2007).

CAR target genes have been recently reported to undergo epigenetic imprinting following CAR activation (Chen et al., 2012; Tien et al., 2015; Li et al., 2016). This prompted us to assess the duration of CAR-mediated induction. Specifically, we investigated whether CAR-mediated hepatic induction is transmitted to the next generation. Such a transmission is conceivable, since it would provide the next generation with an immediate protection against the momentary chemical environment.

We were also motivated by the gaps in developmental toxicity studies for medicinal drugs and for chemicals to be released into the environment. Currently, these studies focus on teratogenic effects. Drugs proven as teratogenic, exemplified by coumarins and valproic acid, are discontinued for the duration of the sensitive stages of pregnancy and replaced by safer alternatives (Schaefer, 2011). Treating pregnant women with drugs with unknown teratogenicity is prohibited or at least discouraged until teratogenicity has been excluded by analyses of sufficient numbers of unintended exposures. Well-known teratogenic environmental chemicals, which should be avoided during pregnancy include TCDD (Couture et al., 1990) and DEHP (Abdul-Ghani et al., 2012).

Less is known about the extent of post-natal and non-teratogenic toxicity, such as described following vinclozolin (Anway et al., 2005), bisphenol A (Salian et al., 2009) or cocaine (Vassoler et al., 2013) exposure. It is also unclear how these toxicities arise. Several mechanisms of “soft inheritance” have been suggested for the transmission of substance-induced phenotypes from one generation to the next, including stable epigenetic mutations and transfer of RNA or substances through seminal fluid, the placenta, or via lactation (Hughes, 2014). The extent and even the sheer existence of such transmission in mammals, including humans, is still contentious (Heard and Martienssen, 2014).

In the following, we report and describe the mechanism of health-relevant phenotypes in the offspring of mice treated well ahead of pregnancy with CAR activators.

Material und Methods

Animals

All animal experiments have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the responsible local ethics committee (Landesuntersuchungsamt Rheinland-Pfalz, reference number 23 177-07/G 13-1-078). All animals were treated humanely with regard to the alleviation of suffering. C57BL/6J were obtained from Envigo (Venray, Netherlands) and CD1 from Janvier (Saint Berthevin, France). Maximally 5 animals per cage were kept on a 12 h light cycle at a temperature of 20–24°C and 45-65% humidity. Females were housed individually beginning with the last 3 days before giving birth.

Induction of mice Newborn mice were injected with a volume of 10 µl/g using 30 G needles, adult mice with 7 or 10 µl/g using 27 G needles. CAR inducers were injected as follows, except if stated otherwise: TCPOBOP (1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene, Sigma-Aldrich, Munich, Germany), 3 mg/kg in corn oil, single i.p. injection to adult and newborn mice; PCB153 (2,2',4,4',5,5'-Hexachlorobiphenyl) 100 mg/kg in corn oil single i.p. injection to adult mice; phenobarbital sodium salt (Sigma-Aldrich) 100 mg/kg in 0.9% NaCl i.p. on 3 consecutive days. Phenobarbital injections to newborn mice were given s.c. and only once 5 days after birth.

Female mice used to obtain F1 offspring were induced one week prior to mating with an uninduced male. Adult males were depleted of spermatozoa by housing with females for one day and injected the following day with TCPOBOP or corn oil. Three, 19 and 31 days after TCPOBOP injection, each male was mated for 4 days with 3 age-matched, untreated, virgin females. The induction was confirmed in all experiments by measuring the expression of the CAR target *Cyp2b10* using a TaqMan assay.

Diets Animals were fed R/M-H Extrudat (#1536, Ssniff, Soest, Germany). For experiments with mice on a high fat diet (HFD, DIO - 60 kJ% fat [Lard], #E15742-34, Ssniff) the control group was fed an appropriate standard diet (SD; DIO - 10 kJ% fat, 11% sucrose „H“, #E15748-04 Ssniff). Food and water were provided *ad libitum* to all animals. In experiments with HFD, the diet was changed from the R/M-H Extrudat to HFD or SD simultaneously with the injection of the inducer to F0 animals.

Embryo transfer Seven-day-old female C57BL/6J mice were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone. Superovulation with PMSG (Pregnant Mare Serum Gonadotropin; 2.5 - 5 IU; i.p.) and hCG (human Chorion Gonadotropin; 2.5 IU; i.p.) was started 3 weeks later with subsequent mating with untreated males for 12 h.

Zygotes were removed 24 h later, were cultured to two cell embryos and transferred to the oviduct of pseudopregnant, narcotized (medetomidin (0.5 µg/g), midazolam (5 µg/g), and fentanyl (0.05 µg/g)) 9-week-old CD1 mice pre-treated with TCPOBOP or corn oil one week in advance. Post-surgery analgesia was conducted using metamizol (100 µg/g, p.o.) and carprofen (4.5 µg/g, s.c.).

Cross-fostering Female C57BL/6J and CD1 mice were treated with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone one week prior to 12 h mating with untreated males of the same strain. E19.5 C57BL/6J F1 mice were delivered by caesarian sections and substituted for up to 50% of CD1 litters. All F1 animals were analyzed 6 days after birth.

Zoxazolamine paralysis assay Paralysis was induced with a single dose of the skeletal muscle relaxant zoxazolamine (Sigma-Aldrich) (175 mg/kg, i.p.) in 10% DMSO in corn oil. Mice were then placed on their backs at a constant temperature of 24°C. Paralysis time was defined and measured between the initiation of paralysis and the time point when the animal was able to right itself.

Glucose tolerance test (GTT) Mice were fasted for 16 h prior to baseline glucose measurement with subsequent glucose injection (10 % Glucose, i.p.). All mice of one sex received the same amount of glucose irrespective of the body weight. The amount of glucose per mouse was calculated as a dose of 2 g/kg of the mean body weight of the mice on standard diet of the respective sex (Ayala et al., 2010). Glucose concentrations were measured using a CONTOUR® XT glucometer (Bayer AG, Leverkusen, Germany) in blood samples collected from the tail 15 min, 30 min, 60 min, and 120 min following the injection.

Serum lipoprotein profile

Serum cholesterol lipoprotein profiles were determined by size-exclusion chromatography as described earlier (Parini et al., 2006; Gil-Pulido et al., 2017). In brief, 5 µl of serum was fractionated using a Superose 6 3.2/300 gel filtration column from GE Healtcare (Uppsala, Sweden) and PBS, pH 7.4 as elution buffer, delivered by a first pump (Waters, Eschborn, Germany 1525 binary pump) at a flow rate of 50 µl/min. The separated lipoproteins were mixed in a T-tube with 50 µl/min cholesterol reagent Chol (Roche, Mannheim, Germany) delivered by a second pump (Waters 1515 pump). Thereafter, the mixture went through a 500 µl reaction coil PEEP tubing (internal diameter 0.75 mm) at 37°C in a post column reaction oven (Waters Temperature Control Module II). Finally, absorption was measured with an UV-VIS detector at 500 nm (Waters 2489 UV/Visible Detector). Total run time for each sample was 60 min. Chromatograms were integrated by Waters Empower 3 software. Very low-density lipoprotein (VLDL), low-density

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations were calculated as products of the area in percent of total cholesterol. Chromatograms for serum lipoprotein separation are shown in Supplementary Fig. 6.

Westernblot

Total protein (20 - 50 µg) lysed in a sample buffer was loaded onto each lane, separated by SDS-PAGE, and transferred to PVDF membrane by tank blotting. The antibodies used were as follows: anti-CYP2B10 (1:3000; #AB9916, Millipore, Darmstadt, Germany), anti-Gapdh (1:20000; #sc-32233, Santa Cruz, Dallas, TX, USA) and anti-mouse (1:10000; #A6782, Sigma-Aldrich), and anti-rabbit (1:5000; #A0545, Sigma-Aldrich) respectively. Bands were visualized by ECL+ detection method (FemtoMax, Rockland, Limerik, PA, USA) using ChemiDoc XRS System with Quantity One (V 4.6.9, Bio-Rad, Munich, Germany) software. Densitometric analysis of the protein bands was done using Image Lab (V 4.1, Bio-Rad).

RNA-Isolation and qPCR

Total hepatic RNA was isolated using TriFast (PeqLab, Erlangen, Germany) and 1 µg was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). *Cyp2b10* expression was quantified using TaqMan assays (primers (Chen et al., 2012), probe: 5'-[FAM]ACGTTCCTCTTCCAGTGCATCAC[BHQ1]-3') on a Step One Plus cycler (Applied Biosystems). 18S rRNA (Mm03928990_g1; Applied Biosystems) was used as an internal control. The expression of *Cyp2b10* was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

RNA Seq data analysis

Three to four-day-old C57BL/6J mice were injected with TCPOBOP (3 mg/kg in corn oil) or corn oil. The hepatic RNA from newborn F0 and F1 mice (5-6 days old) was isolated with RNeasyKit (Qiagen, Hilden, Germany) to meet higher quality needs, while the hepatic RNA from adult F0 and F1 mice (3 month old) was isolated as described in the preceding section. The RNA of 2 to 6 newborn or adult male mice was pooled for the F0 and F1 generation. Hepatic RNA samples were sequenced on Illumina HiSeq 2000 using paired-end library protocol (TruSeq RNA Library Prep Kit, Illumina, San Diego, CA, USA), at the Star SEQ GmbH, Mainz, Germany. We aimed for a sequencing depth of 25 to 50 million reads. Quality of raw sequencing reads was assessed using FastQC (Babraham Bioinformatics, Cambridge, UK) and reads were mapped to the mouse reference genome (gencode release M12 GRCm38.p5) using STAR aligner (v2.5.3a; (Dobin et al., 2013)), with the option "--quantMode GeneCounts" to count the number of reads

per gene. EdgeR (v3.20.1; (Robinson et al., 2010)) was used for differential gene expression analysis. Genes with fold change higher than 2 adjusted by p-value below 0.05 were considered as differentially expressed. Differentially expressed genes obtained from edgeR were uploaded to Ingenuity Pathway Analysis (Qiagen, https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). The core analysis was performed using Ingenuity knowledge as the reference set. The analysis was restricted to human and mouse species and to the differentially expressed genes with a fold change of at least 2. Pathways and upstream regulators were compared among the experimental groups. The sequences and processed files containing the counts in TPM (Transcripts per Kilobase Million) have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002; Barrett et al., 2013) and are accessible through GEO Series accession number GSE109630 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109630).

TCPOBOP extraction from adipose tissue and HPLC-MS/MS analysis

Epididymal fat pads were excised and melted at 60°C. 120 µl of each sample were heated to 50°C in 5 ml of n-Hexan and mixed. Samples were kept at -20°C overnight and centrifuged at 2000 g for 5 min. Supernatant was kept at -20°C overnight and centrifuged again. One ml of the supernatant was dried for 1.5 h in a vacuum concentrator with vapor trap (SC 110 and RVT 100 Savant, Thermo Fisher Scientific, Waltham, MA, USA), dissolved in 100 µl acetonitril/water (1:1) and centrifuged at 13000 g for 2 min. Samples were kept at -20°C. Five µl were taken from the liquid phase and analyzed by HPLC-MS/MS. The separation was performed on an Agilent 1290 UHPLC system equipped with a ZORBAX RRHD SB-C18 column (1.8-µm 2.1 × 50 mm, Agilent technologies, Santa Clara, CA, USA) maintained at 30 °C. The running buffer and the solvent were (A) 5 mM ammonium acetate at pH 6.9 and (B) acetonitrile. The separations were performed at a flow rate of 0.4 ml/min using the following gradients: 50% of solvent B from 0 min to 2 min, linear increase to 95% of solvent B for next 3 min, and kept at 95% of solvent B for another 2 min. To recondition the column, the flow rate was then switched to 0.5 ml/min, and washed with 50% of solvent B for 3 minutes. During the last minute, the flow rate was linearly decreased to the initial value of 0.4 ml/min. The detection was performed on Agilent 6090 triple quadruple mass spectrometer operated in the positive ion mode, using the setting listed in Supplementary Table 4 and Supplementary Table 5.

Statistics

Data was analyzed with GraphPad Prism version 7.00 for Windows. Parametric or non-parametric tests were used according to the results of the Shapiro-Wilk test. Further statistical analysis and numbers of replicates are provided in

the figure legends. Mixed models were fit to the mouse body weight time course data. In detail, the subsets split by diet (SD vs HFD) were fit separately, modeling time and treatment as fixed-effects, and animal as random-effect. Data at time point 0 were excluded for this purpose, as the diet and treatment are applied after this initial measurement. For this purpose, the R statistical software (R Core Team, 2017), (https://www.R-project.org) and the lme4 package (Bates et al., 2015) were used. Throughout the analysis of the manuscript, a significance level alpha = 0.05 was chosen. Since no estimates of effect sizes were available from literature, the target group size was set to 5 animals. With this group size, the power to detect a standardized difference in means of 2 (Cohen's d) is 79% given an alpha level of 5%, as computed with the function power.t.test in the statistics software R. We anticipated higher intragroup variance expected for bodyweight, glucose measurements, and serum lipids, and therefore strove to double the group size. The presented group sizes reflect the additional effect of variabilities in breeding success, survival rates, and sex distributions of litters.

Results

Transmission of *Cyp2b10* induction to F1 and its effects on drug metabolism

The transmission of drug effects to the next generation was first assessed for the prototypical murine CAR-ligand 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and the CAR target gene *Cyp2b10*. Six-day-old F1 offspring of F0 females injected once with 3 mg/kg TCPOBOP one week prior to mating at the age of eight weeks exhibited 747-fold (females) and 517-fold (males) increased *Cyp2b10* mRNA expression compared to the female or male offspring of corn oil (vehicle)-injected females (Fig. 1A, left panel), respectively. The induction of the CYP2B10 protein was confirmed by Western blot (Fig. 1A, right panel). In 8-week-old F1 offspring, the *Cyp2b10* mRNA expression was still increased 3.8-fold in females and 37-fold in males (Fig. 1B). To extend further the interval between the TCPOBOP treatment and the time point of the F1 induction measurement, we injected three-day-old female F0 mice with the same TCPOBOP dose as above and mated them six weeks later with untreated males. The *Cyp2b10* mRNA expression was induced 2.3-fold in the female F1 offspring and 3.7-fold in the male F1 offspring 12 weeks after birth (Fig. 1C). In agreement with previous studies (Chen et al., 2012; Li et al., 2016), we found a robust and long-lasting *Cyp2b10* induction also in F0 females injected with TCPOBOP either as newborns or as adults (Supplementary Fig. 1). Among

other *Cyp* genes, we found a 30-fold induction of *Cyp2c55* in newborn, but not in adult F1 offspring of TCPOBOP-treated females.

To determine the induction transmission through the paternal line, males were treated 3, 19, and 31 days prior to mating, to expose to TCPOBOP spermatozoa/spermatids, spermatocytes, and spermatogonia, respectively. There was a statistically significant induction in offspring generated with TCPOBOP-treated spermatocytes, but its 2-fold magnitude was negligible (Supplementary Fig. 2).

The impact of the transmitted CYP2B10 induction on F1 drug metabolism was assessed as the duration of paralysis upon injection of the muscle relaxant and CYP2B10 substrate zoxazolamine. The paralysis time was defined as the time required by the F1 mice to consciously right themselves. The paralysis time was 55% shorter in the female F1 offspring of F0 females treated with TCPOBOP one week prior to mating compared to the female F1 offspring of control F0 females. An effect of similar magnitude (40%) in the male F1 offspring failed to reach the preset significance level (Fig. 2).

TCPOBOP effects on energy metabolism in the F1 offspring

The 12-week-old F1 offspring of TCPOBOP-treated F0 females gained less weight when set on a standard diet (SD) at the age of 6 weeks, but there was no difference between offspring of treated and untreated females when set on a high fat diet (HFD) (males Fig. 3A, females Supplementary Fig. 3A). The food intake was similar in the F1 offspring of TCPOBOP- and corn oil-treated females (Supplementary Fig. 4). Glucose tolerance was improved only in the male F1 offspring of TCPOBOP-treated F0 females that was kept on HFD diet and only at one time-point (males Fig. 3B, females Supplementary Fig. 3B). The F1 offspring of TCPOBOP-treated F0 females also displayed decreased total cholesterol and HDL levels, whereas LDL and VLDL levels were unchanged (Fig. 3C). These phenotypes mostly resembled the TCPOBOP effects in male F0 mice three days after the injection (Supplementary Fig. 5). (F0 females did not tolerate diet change following TCPOBOP injection). The only exception was that TCPOBOP reduced weight gain irrespective of the diet and that it additionally reduced LDL (Supplementary Fig. 5).

We compared these phenotypes with gene expression changes in livers of male mice induced with TCPOBOP as newborns and analyzed 8 weeks later, as well as in 6-day- and 8-week-old male F1 offspring of TCPOBOP-treated females. Ingenuity Pathway Analysis revealed changes in pathways related to energy metabolism in all TCPOBOP groups included CAR itself and known CAR signaling partners PPARa, LXR , NRF2, FXR, IL6, HNF4a

(Supplementary Table 1, Supplementary Table 2). Among the 5 categories of functions and diseases affected by TCPOBOP were lipid- and drug metabolism (Supplementary Table 3).

Long-term CAR activation in F1 by transmission of the lipophilic CAR ligand via milk

The transmission of TCPOBOP-driven induction to F1 could result from epigenetic modifications in maturating oocytes as early as upon injection of newborn F0 females. Alternatively, it could occur post-conceptionally, either by placenta- or feeding-mediated (via milk) TCPOBOP transfer to the F1 offspring. To distinguish between these possibilities, we conducted embryo transfer experiments. F1 mice conceived by untreated females, but carried to term by foster mothers pre-treated with TCPOBOP, exhibited increased *Cyp2b10* expression. In contrast, F1 mice conceived by TCPOBOP pre-treated donor mice, but carried to term by untreated foster mothers, showed no increase in *Cyp2b10* expression (Fig. 4A). Both of these results suggested a post- rather than pre-conceptional mechanism of induction transmission.

To differentiate between a transplacental (in utero) and a milk-mediated (post-natal) TCPOBOP transfer, we conducted cross-fostering experiments (Fig. 4B). E19.5 F1 offspring of corn oil-treated mice transferred to TCPOBOP pre-treated foster mothers showed 82% of the *Cyp2b10* induction measured in E19.5 F1 offspring of TCPOBOP-treated mice transferred to TCPOBOP pre-treated foster mothers. Conversely, E19.5 F1 offspring of TCPOBOP-treated mice transferred to corn oil-injected foster mothers showed 1% of the *Cyp2b10* induction measured in E19.5 F1 offspring of TCPOBOP-treated mice transferred to TCPOBOP pre-treated foster mothers. These results indicated that a vast majority of the induction occurs via post-natal TCPOBOP transmission. This was additionally confirmed by injecting lactating females with TCPOBOP at different time points (5, 10 or 15 days) after they had given birth. *Cyp2b10* expression strongly increased in the F1 mice suckling for three days after the injection of F0 mothers, as well as in 60-day-old F1 mice, i.e. 6 weeks after weaning (females Fig. 5, males Supplementary Fig. 7). After weaning of F1 offspring, the *Cyp2b10* expression in TCPOBOP pre-treated mothers returned to the level of mothers pre-treated with corn oil (Supplementary Fig. 8).

These results suggested a lactation-mediated transfer of TCPOBOP to F1 offspring, which was confirmed by HPLC-MS/MS analysis of F0 and F1 adipose tissue. The concentration of TCPOBOP, injected to adult F0 females at a dose of 3 mg/kg, was stable in F0 adipose tissue over a period of at least 12 weeks (Fig. 6A, lanes 2 and 9). The TCPOBOP concentration in the adipose tissue of 12-week-old F1 offspring of females induced one week prior to mating was 160-

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

fold lower (Fig. 6A, lane 11). The TCPOBOP concentration in the adipose tissue of F1 offspring was thus similar to that observed in F0 females injected with 100 to 500-times smaller amounts of TCPOBOP (Fig. 6A, lanes 5 and 6). The magnitude of *Cyp2b10* induction in the F1 generation corresponded to that following 1:10th to 100th of the original dose given to F0 (Fig. 6B, lane 4, 5 and 11). Taken together, these results demonstrated that TCPOBOP is transmitted to F1 and that its amount determines the amplitude of *Cyp2b10* induction.

Transmission to F1 may require lipophilicity

Since TCPOBOP is lipophilic, we hypothesized that induction transmission would be a general property of lipophilic compounds. To provide a first verification, we used the lipophilic environmental pollutant and CAR inducer PCB153 (Gahrs et al., 2013; Wahlang et al., 2014). One week after treatment with 100 mg/kg PCB153, adult female mice were mated with untreated males. *Cyp2b10* expression was induced 820-fold in female and 700-fold in male 6-day-old F1 offspring compared to the respective corn oil controls (Fig. 7). We then compared PCB153 with the hydrophilic CAR inducer phenobarbital. Newborn female F0 mice injected once i.p. with 100 mg/kg phenobarbital showed a 70- and 200-fold *Cyp2b10* induction after 24 h in females and males, respectively. Six weeks later, the induction declined to 3.3-fold in females and 1.6-fold in males (Supplementary Fig. 9A). Newborn F1 offspring of these phenobarbital-treated mice showed no *Cyp2b10* induction (Supplementary Fig. 9B).

Discussion

We demonstrate that long lasting, lipophilic chemicals applied far ahead of pregnancy are transmitted via milk and evoke health-relevant phenotypes in the adult F1 offspring. We show it for two ligands of the xenosensing nuclear receptor CAR, but the mechanism likely applies to any other receptor modifiable by lipophilic chemicals. The resulting F1 phenotypes include subtle changes of energy and drug metabolism, which may manifest clinically only later in life, or under specific conditions, such as drug treatments. Due to the long-term accumulation in adipose tissue, the ligand transmission to F1, exemplified by TCPOBOP, takes place even if the treatment has been terminated far ahead of conception. These findings are of importance for the management of chemicals released into the environment, for the development of new drugs, and for planning and management of pregnancies.

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Mechanism

The latest literature suggests persisting epigenetic changes in the promoters of CAR-target genes including *Cyp2b10* to be responsible for prolonged induction within the F0 generation in the liver (Chen et al., 2012; Peng and Zhong, 2015; Tien et al., 2015; Li et al., 2016; Piekos et al., 2017; Tien et al., 2017; Lodato et al., 2018). Our work indicates that at least some of these effects may have been driven by activator accumulation rather than by stable DNA modifications.

The involvement of a physical ligand transfer in the induction transmission is supported by the detection in F1 of TCPOBOP (this study) and of PCB153 (Gallenberg and Vodicnik, 1987; Ring et al., 1990). It is also consistent with the long-term accumulation of these compounds in F0 adipose tissue (Poland et al., 1980; Gallenberg and Vodicnik, 1987; Gallenberg et al., 1990; Ring et al., 1990; Smith et al., 1993), confirmed in this study. Adipose tissue undergoes mobilization upon lactation (Lederman, 2004). The resulting TCPOBOP transfer to F1 is in accordance with the absence of *Cyp2b10* induction in mothers after weaning. The mobilization also reduces the PCB153 burden in F0 mothers, while the chemical becomes detectable in the F1 offspring (Gallenberg and Vodicnik, 1987). Milk is the main vehicle of transmission, as demonstrated previously for PCB153 (Gallenberg and Vodicnik, 1987) and in this study for TCPOBOP. Accordingly, we found a nearly maximal *Cyp2b10* induction in caesarian section-delivered F1 offspring of TCPOBOP-naïve mothers transferred to TCPOBOP pre-treated foster (i.e. lactating) mothers. Induction also occurred also upon injecting lactating females with TCPOBOP. Significantly, PCB153 has been found in human breast milk samples (Lignell et al., 2009).

In comparison with the amounts of TCPOBOP transferred via milk, the transplacental transmission was minimal, judging from the negligible induction in F1 offspring of TCPOBOP-treated females transferred to TCPOBOP-naïve foster mothers for lactation. Similarly, PCB153 does not cross placenta, which has been attributed to the strong binding to VLDL (Gallenberg and Vodicnik, 1987). TCPOBOP transfer via fur licking could not have contributed to induction transmission, since allo-grooming starts only several days after our time point of F1 induction analysis (Williams and Scott, 1953; Fox, 1965). We excluded the possibility of pre-conceptional “imprinting” of induction by TCPOBOP by embryo transfer experiments and we did not detect any effects in F1 following the less lipophilic phenobarbital.

Non-genetic transfer of phenotypes originating from a treatment *prior to pregnancy* has been attributed to epigenetic marks in the gametes (Anway et al., 2005), to somatic reprogramming following *in utero* exposure (Iqbal et al., 2015),

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

or to RNA transfer via sperm cells (Rodgers et al., 2015). Our work demonstrates that such phenotypes may arise via accumulation of chemicals and their transfer to the next generation. The possible routes are placenta (Jeong et al., 2018), seminal fluid (Bromfield et al., 2014), and milk, as demonstrated here using a specific ligand-receptor pair.

Metabolic effects

The CYP2B10 induction in the F1 generation decreased efficacy of the muscle relaxant zoxazolamine. The transcriptional targets of human CAR *CYP2B6*, *CYP3A4*, and *UGT1A1* (Park et al., 2016) metabolize a majority of clinically used drugs including antineoplastics, anesthetics, opioids and antiretrovirals (Wang and Tompkins, 2008; Hedrich et al., 2016). It is tempting to speculate that long-lasting CAR ligands are responsible for inter-individual expression variability of these targets that cannot be explained by genetic variants (Zanger et al., 2001; Peng and Zhong, 2015; Hedrich et al., 2016), although further experiments are needed to validate this hypothesis. This variability further complicates drug dose finding, especially in newborns, or may cause toxicities (Tayman et al., 2011; Pauwels and Allegaert, 2016).

TCPOBOP-mediated effects on F1 energy metabolism included a reduction of body weight gain, improved glucose tolerance during high fat diet and reduced serum lipids. They are consistent with a protective role of CAR induction against metabolic syndrome-like phenotypes in F0 (Gao et al., 2009; Kobayashi et al., 2015). The reduction of body weight gain in F0 and F1 was independent of food intake supporting the previously reported higher energy expenditure upon CAR activation in the brown adipose tissue (Gao et al., 2009). The observed F1 phenotypes match those in F0 animals in terms of directionality. The subtle differences are most likely due to the lower exposure of the F1 to TCPOBOP compared with the F0.

The phenotypes were in line with transcriptomic changes. Besides drug metabolism, TCPOBOP affected regulators of pathways involved in the energy homeostasis of newborn and adult F0 mice as well as newborn and adult F1 mice. PPARa, FXR and LXR have an impact on energy metabolism and the pathogenesis of fatty liver disease (Kersten and Stienstra, 2017; Li et al., 2017; Tanaka et al., 2017). IL6 plays a role in type 2 diabetes (Phosat et al., 2017) and HNF4a in hereditary diabetes (Yamagata et al., 1996). Nrf2 is involved in glucose resistance and dyslipidemia (Xu et al., 2015; Zhang et al., 2015b). Lipid metabolism was one of the top three regulated pathways 60 days after TCPOBOP treatment of F0 animals in a previous study (2016).

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Significance for chemical safety and pregnancy management

Termination of therapy 6-24 months ahead of pregnancy is presently recommended only for a handful of long-term accumulating drugs, including cytostatics, radioiodine, vitamin K antagonists, amiodarone, leflunomide, methotrexate and retinoids. These drugs are either teratogenic, or they cause adverse effects manifesting immediately after birth, exemplified by hypothyroidism after amiodarone (Magee et al., 1995).

Our work strongly suggests that these recommendations should be expanded to other drugs and extended to the avoidance of certain environmental chemicals. Indeed, long-term accumulating chemicals, exemplified here by TCPOBOP and PCB153 (Schecter et al., 2010; Whitehead et al., 2013; Pozo et al., 2017), may be transmitted to F1 and induce health-relevant phenotypes even if treatment has been terminated far ahead of conception. Such phenotypes may remain undetected, since developmental toxicology currently focuses on teratogenic effects. These phenotypes are *more subtle and manifest later in life*. It is likely that such unapparent phenotypes arise in F1 after the transmission of lipophilic ligands of other receptors. This could and should be assessed for both approved and investigational drugs and chemicals known or suspected of accumulation in adipose tissue.

Such drugs and chemicals can be identified by a combination of small molecular weight, high logP and pKa<7 (Moor et al., 1992), which also correlate with the likelihood of transfer via milk (Howard and Lawrence, 2001; Kar and Roy, 2013). Highly lipophilc drugs (logP>6) include the CAR ligand atorvastatin (Rezen et al., 2017), as well as montelukast, raloxifene, cinacalcet, fexofenadine, repaglinide, telmisartan, adapalene, terbinafine, dutasteride, and levothyroxine (Zhong et al., 2013). Many common environmental pollutants are lipophilic (logP>3) CAR ligands (e.g. DDT (Kamata et al., 2018), permethrin (Kublbeck et al., 2011), chlordane (Kamata et al., 2018), dibutylphthalate (Wyde et al., 2005), dieldrin (Wei et al., 2002), methoxychlor (Kamata et al., 2018), pyren (Zhang et al., 2015a) and toxaphen (Wei et al., 2002)), which cross the placenta or are transferred via milk (Campoy et al., 2001; Skopp et al., 2002; Cok et al., 2012; Corcellas et al., 2012; Limon-Miro et al., 2017; Muller et al., 2017; Wang et al., 2017; Yasmeen et al., 2017). The lipophilic pollutants and CAR modulators BPA, DEHP (DeKeyser et al., 2011) exhibit not only multi- but even transgenerational (beyond F1) effects (Ziv-Gal et al., 2015; Pocar et al., 2017). The assessment of F1 effects could start with transcriptional profiling of appropriate animal models.

Acknowledgements

The authors thank Professor M. Michel (Department of Pharmacology, University Medical Center Mainz) and Dr. R. Kaiser (Boehringer Ingelheim Pharma GmbH & Co. KG) for comments to the manuscript. The authors gratefully acknowledge the computing time granted on the supercomputer Mogon at Johannes Gutenberg University Mainz (hpc.uni-mainz.de).

Author Contributions

Participated in research design: Dietrich, Baumgart, Eshkind, Butt, Musheev, Niehrs, Wojnowski, Mathäs

Conducted experiments: Dietrich, Baumgart, Eshkind, Reuter, Gödtel-Armbrust, Butt, Musheev, Grosser, Mathäs

Contributed new reagents or analytic tools: Butt, Musheev, Niehrs

Performed data analysis: Dietrich, Butt, Musheev, Marini, More, Mathäs

Wrote or contributed to the writing of the manuscript: Wojnowski, Mathäs

References

Abdul-Ghani S, Yanai J, Abdul-Ghani R, Pinkas A, and Abdeen Z (2012) The teratogenicity and behavioral teratogenicity of di(2-ethylhexyl) phthalate (DEHP) and di-butyl phthalate (DBP) in a chick model. *Neurotoxicol Teratol* **34:**56-62.

Anway MD, Cupp AS, Uzumcu M, and Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science (New York, NY* **308:**1466-1469.

Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH, McGuinness OP, and Consortium NIHMMPC (2010) Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech* **3:**525-534.

Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, and Soboleva A (2013) NCBI GEO: archive for functional genomics data sets--update. *Nucleic acids research* **41:**D991-995.

Bates D, Machler M, Bolker BM, and Walker SC (2015) Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw* **67:**1-48.

Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, and Robertson SA (2014) Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America* **111:**2200-2205.

Campoy C, Jimenez M, Olea-Serrano MF, Moreno-Frias M, Canabate F, Olea N, Bayes R, and Molina-Font JA (2001) Analysis of organochlorine pesticides in human milk: preliminary results. *Early Hum Dev* **65 Suppl:**S183-190.

Chen WD, Fu X, Dong B, Wang YD, Shiah S, Moore DD, and Huang W (2012) Neonatal activation of the nuclear receptor CAR results in epigenetic memory and permanent change of drug metabolism in mouse liver. *Hepatology*.

Cok I, Mazmanci B, Mazmanci MA, Turgut C, Henkelmann B, and Schramm KW (2012) Analysis of human milk to assess exposure to PAHs, PCBs and organochlorine pesticides in the vicinity Mediterranean city Mersin, Turkey. *Environ Int* **40:**63-69.

Corcellas C, Feo ML, Torres JP, Malm O, Ocampo-Duque W, Eljarrat E, and Barcelo D (2012) Pyrethroids in human breast milk: occurrence and nursing daily intake estimation. *Environ Int* **47:**17-22.

Couture LA, Abbott BD, and Birnbaum LS (1990) A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin: recent advances toward understanding the mechanism. *Teratology* **42:**619-627.

DeKeyser JG, Laurenzana EM, Peterson EC, Chen T, and Omiecinski CJ (2011) Selective phthalate activation of naturally occurring human constitutive androstane receptor splice variants and the pregnane X receptor. *Toxicological sciences : an official journal of the Society of Toxicology* **120:**381-391.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, and Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)* **29:**15-21.

Edgar R, Domrachev M, and Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research* **30:**207-210.

Fox WM (1965) Reflex-ontogeny and behavioural development of the mouse. *Anim Behav* **13:**234-241.

Gahrs M, Roos R, Andersson PL, and Schrenk D (2013) Role of the nuclear xenobiotic receptors CAR and PXR in induction of cytochromes P450 by non-dioxinlike polychlorinated biphenyls in cultured rat hepatocytes. *Toxicol Appl Pharmacol* **272:**77-85.

Gallenberg LA, Ring BJ, and Vodicnik MJ (1990) The influence of time of maternal exposure to 2,4,5,2',4',5'-hexachlorobiphenyl on its accumulation in their nursing offspring. *Toxicol Appl Pharmacol* **104:**1-8.

Gallenberg LA and Vodicnik MJ (1987) Potential mechanisms for redistribution of polychlorinated biphenyls during pregnancy and lactation. *Xenobiotica* **17:**299-310.

Gao J, He J, Zhai Y, Wada T, and Xie W (2009) The constitutive androstane receptor is an anti-obesity nuclear receptor that improves insulin sensitivity. *The Journal of biological chemistry* **284:**25984-25992.

Gil-Pulido J, Cochain C, Lippert MA, Schneider N, Butt E, Amezaga N, and Zernecke A (2017) Deletion of Batf3-dependent antigen-presenting cells does not affect atherosclerotic lesion formation in mice. *PloS one* **12:**e0181947.

Heard E and Martienssen RA (2014) Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* **157:**95-109.

Hedrich WD, Hassan HE, and Wang H (2016) Insights into CYP2B6-mediated drug-drug interactions. *Acta Pharm Sin B* **6:**413-425.

Howard CR and Lawrence RA (2001) Xenobiotics and breastfeeding. *Pediatr Clin North Am* **48:**485-504.

Hughes V (2014) Epigenetics: The sins of the father. *Nature* **507:**22-24.

Iqbal K, Tran DA, Li AX, Warden C, Bai AY, Singh P, Wu X, Pfeifer GP, and Szabo PE (2015) Deleterious effects of endocrine disruptors are corrected in the mammalian germline by epigenome reprogramming. *Genome biology* **16:**59.

Jeong Y, Lee S, Kim S, Park J, Kim HJ, Choi G, Choi S, Kim S, Kim SY, Kim S, Choi K, and Moon HB (2018) Placental transfer of persistent organic pollutants and feasibility using the placenta as a non-invasive biomonitoring matrix. *Sci Total Environ* **612:**1498-1505.

Kamata R, Nakajima D, and Shiraishi F (2018) Agonistic effects of diverse xenobiotics on the constitutive androstane receptor as detected in a recombinant yeast-cell assay. *Toxicol In Vitro* **46:**335-349.

Kar S and Roy K (2013) Prediction of Milk/Plasma Concentration Ratios of Drugs and Environmental Pollutants Using In Silico Tools: Classification and Regression Based QSARs and Pharmacophore Mapping. *Mol Inform* **32:**693-705.

Kersten S and Stienstra R (2017) The role and regulation of the peroxisome proliferator activated receptor alpha in human liver. *Biochimie* **136:**75-84.

Kobayashi K, Hashimoto M, Honkakoski P, and Negishi M (2015) Regulation of gene expression by CAR: an update. *Archives of toxicology* **89:**1045-1055.

Kublbeck J, Laitinen T, Jyrkkarinne J, Rousu T, Tolonen A, Abel T, Kortelainen T, Uusitalo J, Korjamo T, Honkakoski P, and Molnar F (2011) Use of comprehensive screening methods to detect selective human CAR activators. *Biochemical pharmacology* **82:**1994-2007.

Lederman SA (2004) Influence of lactation on body weight regulation. *Nutr Rev* **62:**S112-119.

Li CY, Cheng SL, Bammler TK, and Cui JY (2016) Editor's Highlight: Neonatal Activation of the Xenobiotic-Sensors PXR and CAR Results in Acute and Persistent Down-regulation of PPARalpha-Signaling in Mouse Liver. *Toxicological sciences : an official journal of the Society of Toxicology* **153:**282-302.

Li G, Brocker CN, Xie C, Yan T, Noguchi A, Krausz KW, Xiang R, and Gonzalez FJ (2017) Hepatic PPARalpha mediates the major metabolic effects of Wy-14643. *J Gastroenterol Hepatol*.

Lignell S, Aune M, Darnerud PO, Cnattingius S, and Glynn A (2009) Persistent organochlorine and organobromine compounds in mother's milk from Sweden 1996-2006: compound-specific temporal trends. *Environ Res* **109:**760-767.

Limon-Miro AT, Aldana-Madrid ML, Alvarez-Hernandez G, Antunez-Roman LE, Rodriguez-Olibarria G, and Valencia Juillerat ME (2017) Breast milk intake and mother to infant pesticide transfer measured by deuterium oxide dilution in agricultural and urban areas of Mexico. *Chemosphere* **181:**682-689.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:**402-408.

Lodato NJ, Rampersaud A, and Waxman DJ (2018) Impact of CAR Agonist Ligand TCPOBOP on Mouse Liver Chromatin Accessibility. *Toxicological sciences : an official journal of the Society of Toxicology* **164:**115-128.

Magee LA, Downar E, Sermer M, Boulton BC, Allen LC, and Koren G (1995) Pregnancy outcome after gestational exposure to amiodarone in Canada. *American journal of obstetrics and gynecology* **172:**1307-1311.

Moor MJ, Steiner SH, Jachertz G, and Bickel MH (1992) Adipose tissue distribution and chemical structure of basic lipophilic drugs: desipramine, N-acetyl desipramine, and haloperidol. *Pharmacol Toxicol* **70:**121-124.

Moreau A, Vilarem MJ, Maurel P, and Pascussi JM (2008) Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. *Molecular pharmaceutics* **5:**35-41.

Muller MH, Polder A, Brynildsrud OB, Karimi M, Lie E, Manyilizu WB, Mdegela RH, Mokiti F, Murtadha M, Nonga HE, Skaare JU, and Lyche JL (2017) Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in human breast milk and associated health risks to nursing infants in Northern Tanzania. *Environ Res* **154:**425-434.

Parini P, Johansson L, Broijersen A, Angelin B, and Rudling M (2006) Lipoprotein profiles in plasma and interstitial fluid analyzed with an automated gel-filtration system. *Eur J Clin Invest* **36:**98-104.

Park S, Cheng SL, and Cui JY (2016) Characterizing drug-metabolizing enzymes and transporters that are bona fide CAR-target genes in mouse intestine. *Acta Pharm Sin B* **6:**475-491.

Pauwels S and Allegaert K (2016) Therapeutic drug monitoring in neonates. *Arch Dis Child* **101:**377-381.

Peng L and Zhong X (2015) Epigenetic regulation of drug metabolism and transport. *Acta Pharm Sin B* **5:**106-112.

Phosat C, Panprathip P, Chumpathat N, Prangthip P, Chantratita N, Soonthornworasiri N, Puduang S, and Kwanbunjan K (2017) Elevated C-reactive protein, interleukin 6, tumor necrosis factor alpha and glycemic load associated with type 2 diabetes mellitus in rural Thais: a cross-sectional study. *BMC Endocr Disord* **17:**44.

Piekos S, Pope C, Ferrara A, and Zhong XB (2017) Impact of Drug Treatment at Neonatal Ages on Variability of Drug Metabolism and Drug-drug Interactions in Adult Life. *Curr Pharmacol Rep* **3:**1-9.

Pocar P, Fiandanese N, Berrini A, Secchi C, and Borromeo V (2017) Maternal exposure to di(2-ethylhexyl)phthalate (DEHP) promotes the transgenerational inheritance of adult-onset reproductive dysfunctions through the female germline in mice. *Toxicol Appl Pharmacol* **322:**113-121.

Poland A, Mak I, Glover E, Boatman RJ, Ebetino FH, and Kende AS (1980) 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity. *Molecular pharmacology* **18:**571-580.

Pozo K, Oyola G, Estellano VH, Harner T, Rudolph A, Prybilova P, Kukucka P, Audi O, Klanova J, Metzdorff A, and Focardi S (2017) Persistent Organic Pollutants (POPs) in the atmosphere of three Chilean cities using passive air samplers. *Sci Total Environ* **586:**107-114.

Prakash C, Zuniga B, Song CS, Jiang S, Cropper J, Park S, and Chatterjee B (2015) Nuclear Receptors in Drug Metabolism, Drug Response and Drug Interactions. *Nucl Receptor Res* **2**.

R Core Team (2017) R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria.

Rezen T, Hafner M, Kortagere S, Ekins S, Hodnik V, and Rozman D (2017) Rosuvastatin and Atorvastatin Are Ligands of the Human Constitutive Androstane Receptor/Retinoid X Receptor alpha Complex. *Drug metabolism and disposition: the biological fate of chemicals* **45:**974-976.

Ring BJ, Seitz KR, Gallenberg LA, and Vodicnik MJ (1990) The effect of diet and litter size on the elimination of 2,4,5,2',4',5'-[14C]hexachlorobiphenyl from lactating mice. *Toxicol Appl Pharmacol* **104:**9-16.

Robinson MD, McCarthy DJ, and Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26:**139-140.

Rodgers AB, Morgan CP, Leu NA, and Bale TL (2015) Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proceedings of the National Academy of Sciences of the United States of America* **112:**13699-13704.

Salian S, Doshi T, and Vanage G (2009) Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring. *Life sciences* **85:**742-752.

Schaefer C (2011) Drug safety in pregnancy: utopia or achievable prospect? Risk information, risk research and advocacy in Teratology Information Services. *Congenit Anom (Kyoto)* **51:**6-11.

Schecter A, Colacino J, Haffner D, Patel K, Opel M, Papke O, and Birnbaum L (2010) Perfluorinated compounds, polychlorinated biphenyls, and organochlorine pesticide contamination in composite food samples from Dallas, Texas, USA. *Environmental health perspectives* **118:**796-802.

Skopp S, Oehme M, and Furst P (2002) Enantiomer ratios, patterns and levels of toxaphene congeners in human milk from Germany. *J Environ Monit* **4:**389-394.

Smith G, Henderson CJ, Parker MG, White R, Bars RG, and Wolf CR (1993) 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene, an extremely potent modulator of mouse hepatic cytochrome P-450 gene expression. *The Biochemical journal* 289 (Pt 3):807-813.

Tanaka N, Aoyama T, Kimura S, and Gonzalez FJ (2017) Targeting nuclear receptors for the treatment of fatty liver disease. *Pharmacol Ther* 179:142-157.

Tayman C, Rayyan M, and Allegaert K (2011) Neonatal pharmacology: extensive interindividual variability despite limited size. *J Pediatr Pharmacol Ther* 16:170-184.

Tien YC, Liu K, Pope C, Wang P, Ma X, and Zhong XB (2015) Dose of Phenobarbital and Age of Treatment at Early Life are Two Key Factors for the Persistent Induction of Cytochrome P450 Enzymes in Adult Mouse Liver. *Drug metabolism and disposition: the biological fate of chemicals* 43:1938-1945.

Tien YC, Piekos SC, Pope C, and Zhong XB (2017) Phenobarbital Treatment at a Neonatal Age Results in Decreased Efficacy of Omeprazole in Adult Mice. *Drug metabolism and disposition: the biological fate of chemicals*.

Vassoler FM, White SL, Schmidt HD, Sadri-Vakili G, and Pierce RC (2013) Epigenetic inheritance of a cocaine-resistance phenotype. *Nature neuroscience* 16:42-47.

Wahlang B, Falkner KC, Clair HB, Al-Eryani L, Prough RA, States JC, Coslo DM, Omiecinski CJ, and Cave MC (2014) Human receptor activation by aroclor 1260, a polychlorinated biphenyl mixture. *Toxicological sciences : an official journal of the Society of Toxicology* 140:283-297.

Wang H and Tompkins LM (2008) CYP2B6: new insights into a historically overlooked cytochrome P450 isozyme. *Current drug metabolism* 9:598-610.

Wang X, Sheng N, Cui R, Zhang H, Wang J, and Dai J (2017) Gestational and lactational exposure to di-isobutyl phthalate via diet in maternal mice decreases testosterone levels in male offspring. *Chemosphere* 172:260-267.

Wei P, Zhang J, Dowhan DH, Han Y, and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *The pharmacogenomics journal* 2:117-126.

Whitehead TP, Ward MH, Colt JS, Nishioka MG, Buffler PA, Rappaport SM, and Metayer C (2013) Determinants of polychlorinated biphenyls in dust from homes in California, USA. *Environ Sci Process Impacts* 15:339-346.

Williams E and Scott JP (1953) The Development of Social Behavior Patterns in the Mouse, in Relation to Natural Periods. *Behaviour* 6:35-65.

Wyde ME, Kirwan SE, Zhang F, Laughter A, Hoffman HB, Bartolucci-Page E, Gaido KW, Yan B, and You L (2005) Di-n-butyl phthalate activates constitutive androstane receptor and pregnane X receptor and enhances the expression of steroid-metabolizing enzymes in the liver of rat fetuses. *Toxicological sciences : an official journal of the Society of Toxicology* 86:281-290.

Xu J, Donepudi AC, More VR, Kulkarni SR, Li L, Guo L, Yan B, Chatterjee T, Weintraub N, and Slitt AL (2015) Deficiency in Nrf2 transcription factor decreases adipose tissue mass and hepatic lipid accumulation in leptin-deficient mice. *Obesity (Silver Spring)* 23:335-344.

Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, and Bell GI (1996) Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature* 384:458-460.

Yamazaki Y, Kakizaki S, Horiguchi N, Sohara N, Sato K, Takagi H, Mori M, and Negishi M (2007) The role of the nuclear receptor constitutive androstane receptor in the pathogenesis of non-alcoholic steatohepatitis. *Gut* **56:**565-574.

Yang H and Wang H (2014) Signaling control of the constitutive androstane receptor (CAR). *Protein Cell* **5:**113-123.

Yasmeen H, Qadir A, Mumtaz M, Eqani S, Syed JH, Mahmood A, Jamil N, Nazar F, Ali H, Ahmad MS, Tanveer ZI, and Zhang G (2017) Risk profile and health vulnerability of female workers who pick cotton by organanochlorine pesticides from southern Punjab, Pakistan. *Environ Toxicol Chem* **36:**1193-1201.

Zanger UM, Fischer J, Raimundo S, Stuven T, Evert BO, Schwab M, and Eichelbaum M (2001) Comprehensive analysis of the genetic factors determining expression and function of hepatic CYP2D6. *Pharmacogenetics* **11:**573-585.

Zhang XJ, Shi Z, Lyv JX, He X, Englert NA, and Zhang SY (2015a) Pyrene is a Novel Constitutive Androstane Receptor (CAR) Activator and Causes Hepatotoxicity by CAR. *Toxicological sciences : an official journal of the Society of Toxicology* **147:**436-445.

Zhang Z, Zhou S, Jiang X, Wang YH, Li F, Wang YG, Zheng Y, and Cai L (2015b) The role of the Nrf2/Keap1 pathway in obesity and metabolic syndrome. *Rev Endocr Metab Disord* **16:**35-45.

Zhong HA, Mashinson V, Woolman TA, and Zha M (2013) Understanding the molecular properties and metabolism of top prescribed drugs. *Curr Top Med Chem* **13:**1290-1307.

Ziv-Gal A, Wang W, Zhou C, and Flaws JA (2015) The effects of in utero bisphenol A exposure on reproductive capacity in several generations of mice. *Toxicol Appl Pharmacol* **284:**354-362.

Footnotes

This work was supported by the German Research Foundation (DFG) grant MA6821/1-1 (to M. Mathäs), by internal funding of the Johannes Gutenberg-University Mainz (to L. Wojnowski), by the Interdisciplinary Center for Clinical Research (IZKF Würzburg) funding Z-4/123 (to E. Butt) and by the International PhD Programme Mainz (to K. Dietrich and P. More).

Data is in part included in the doctoral thesis of Karin Dietrich

Legends for Figures

Fig. 1 *Cyp2b10* induction in the F1 offspring driven by a single maternal, pre-conceptional TCPOBOP injection.

Hepatic *Cyp2b10* mRNA expression was determined by q-PCR and ΔΔct-method. Adult (A, B) or 3-day-old (C) female F0 mice were injected i.p. with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone. C expression in F1 mice was assessed 6 days (A), 8 weeks (B) or 12-weeks (C) after birth. Data are presented as mean fold-expression changes ± SEM over the mean male corn oil group (n = 4-10), which were analyzed by t-test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (***,$P < 0.001$; *,$P < 0.05$). A. (right panel) Western blot of hepatic CYP2B10 expression of 4 male 6-day-old F1 mice born from females injected with TCPOBOP or with corn oil 1 week prior to mating. Gapdh expression served as a loading control.

Fig. 2 Effects of F0 TCPOBOP treatment on the zoxazolamine-induced paralysis in F1 mice

Female F0 mice were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone one week prior to mating with untreated males. Shown is the duration of zoxazolamine (175 mg/kg, i.p.)- induced paralysis time in eight-week-old F1 offspring. Data are presented as mean ± SEM (n = 5-14) and were analyzed by t-test. Statistically significant differences are indicated by asterisks (*** $P < 0.001$).

Fig. 3 Body weight, glucose tolerance, and serum lipids in the F1 offspring following a single, pre-conceptional TCPOBOP injection.

A. 6-week-old male offspring of females injected with TCPOBOP (3 mg/kg) in corn oil (solid line) or with corn oil alone (dotted line) one week prior to mating with untreated males were set on high fat diet (HFD) (blue) or standard diet (SD) (black) for nine weeks. Statistically significant differences in body weight were analyzed using a mixed model and treatment related differences are indicated by asterisks (***, $P < 0.001$). Data are presented as mean ± SEM. n = 6-11 **B.** Following week nine, all mice were fasted for 16 h and challenged with glucose (2 g/kg) according to the mean body weight of male animals on standard diet (same dose for each animal). Shown are mean concentrations ± SEM in male offspring. Blood glucose was analyzed by t-test or Mann-Whitney test with Bonferroni correction for four time points (0 min, 15 min, 30 min, 60 min). Statistically significant differences are indicated by asterisks (*,$P < 0.05$). n = 6-9 **C.** Total cholesterol, VLDL, LDL, and HDL were analyzed using HPLC

in 12-week-old offspring on normal diet. Mice were fasted for 16 h before the analysis. Serum lipid concentrations were analyzed by t-test with Bonferroni correction for four parameters, statistically significant differences are indicated by asterisks (***,P < 0.001; **,P < 0.01; *,P < 0.05). Data are presented as mean ± SEM. n = 5-9. Example serum lipid profiles are shown in Supplementary Fig. 6.

Fig. 4 *Cyp2b10* mRNA expression in the embryo-transferred or cross-fostered F1 offspring.

A. Embryo transfer: Donor C57BL/6J females were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone and super-ovulated and mated with untreated males 3 weeks later. Two-cell embryos were transferred to CD1 recipients pre-treated one week earlier with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone, and carried to term (n = 29-50). **B.** Cross-fostering: C57BL/6J and CD1 females were injected with TCPOBOP (3 mg/kg) in corn oil or with corn oil alone, and mated with untreated males one week later. Following caesarian section on gestational day 19.5, F1 C57BL/6J offspring mice (n = 4-18) were substituted for up to 50% of litters nursed by CD1 mice. The hepatic *Cyp2b10* mRNA expression was determined in six-day-old F1 animals by q-PCR and ΔΔct-method and was analyzed by Kruskal-Wallis test and Dunn's post hoc-test. Statistically significant differences over the control "corn oil to corn oil" are indicated by asterisks (***,P < 0.001; *,P < 0.05). Data are presented as mean ± SEM.

Fig. 5 TCPOBOP transfer via lactation initiated at 3 time points after birth

Lactating F0 females were treated with TCPOBOP on day 3, 10 or 15 after having given birth. Hepatic *Cyp2b10* mRNA expression was analyzed in female F1 newborns 3 days after maternal TCPOBOP treatment, n=2-5 (A) or in 60-day-old female F1 mice, n = 5-10 (B). Shown are mean expression changes over mean male controls ± SEM determined by q-PCR and ΔΔct-method, which were analyzed by t-test or Mann-Whitney test. Statistically significant differences are indicated by asterisks (***,P < 0.001; **,P < 0.01; *,P < 0.05). Similar results were obtained for male offspring (Supplementary Fig. 7).

Fig. 6 TCPOBOP concentrations in the adipose tissue and the corresponding hepatic *Cyp2b10* induction.

A. TCPOBOP was detected by HPLC-MS/MS. TCPOBOP was extracted from 120 µl of melted epididymal adipose tissue of mice treated at the age of 11 weeks with different TCPOBOP concentrations (0 mg/kg, 3 mg/kg, 0.6 mg/kg, 0.3 mg/kg, 0.03 mg/kg, 0.006 mg/kg, 0.003 mg/kg) 3 days prior to analysis, and with 3 mg/kg 12 weeks prior to analysis. F1 offspring of F0 female mice injected with TCPOBOP (3 mg/kg) and mated one week later was analyzed

12 weeks after birth. Shown are mean TCPOBOP concentrations ± SEM (n=4). B. *Cyp2b10* mRNA expression changes over the respective time point- and generation-mated, corn oil-injected controls in livers of mice shown in (A) was determined by qPCR and ΔΔct-method. Data are presented as mean fold-expression changes ± SEM.

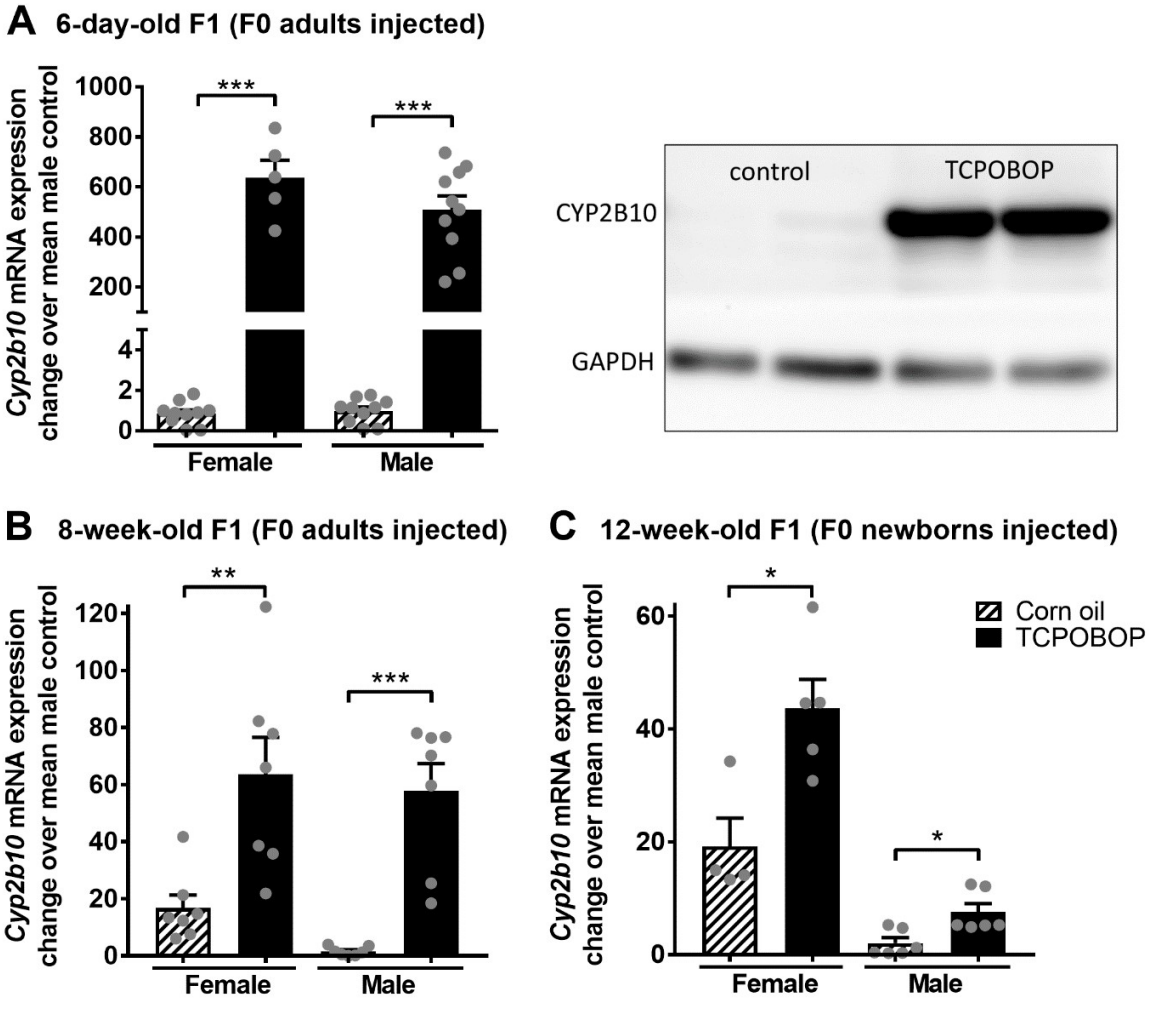
Fig. 7 *Cyp2b10* induction in the F1-generation by PCB153.

Seven-week-old adult F0 female mice were injected once with PCB153 (100 mg/kg) or vehicle (corn oil). After one week, they were mated with untreated males. *Cyp2b10* expression was assessed in 6-day-old F1 offspring. Shown are mean expression changes over the mean of male controls ± SEM determined by q-PCR and ΔΔct-method and analyzed by t-test. Statistically significant differences are indicated by asterisks (***,$P < 0.001$).

DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

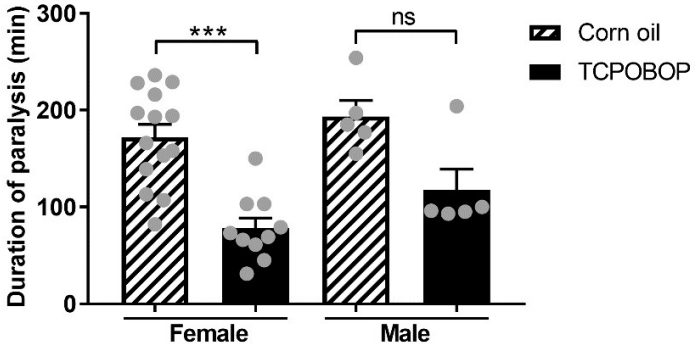
Figures

Fig. 1



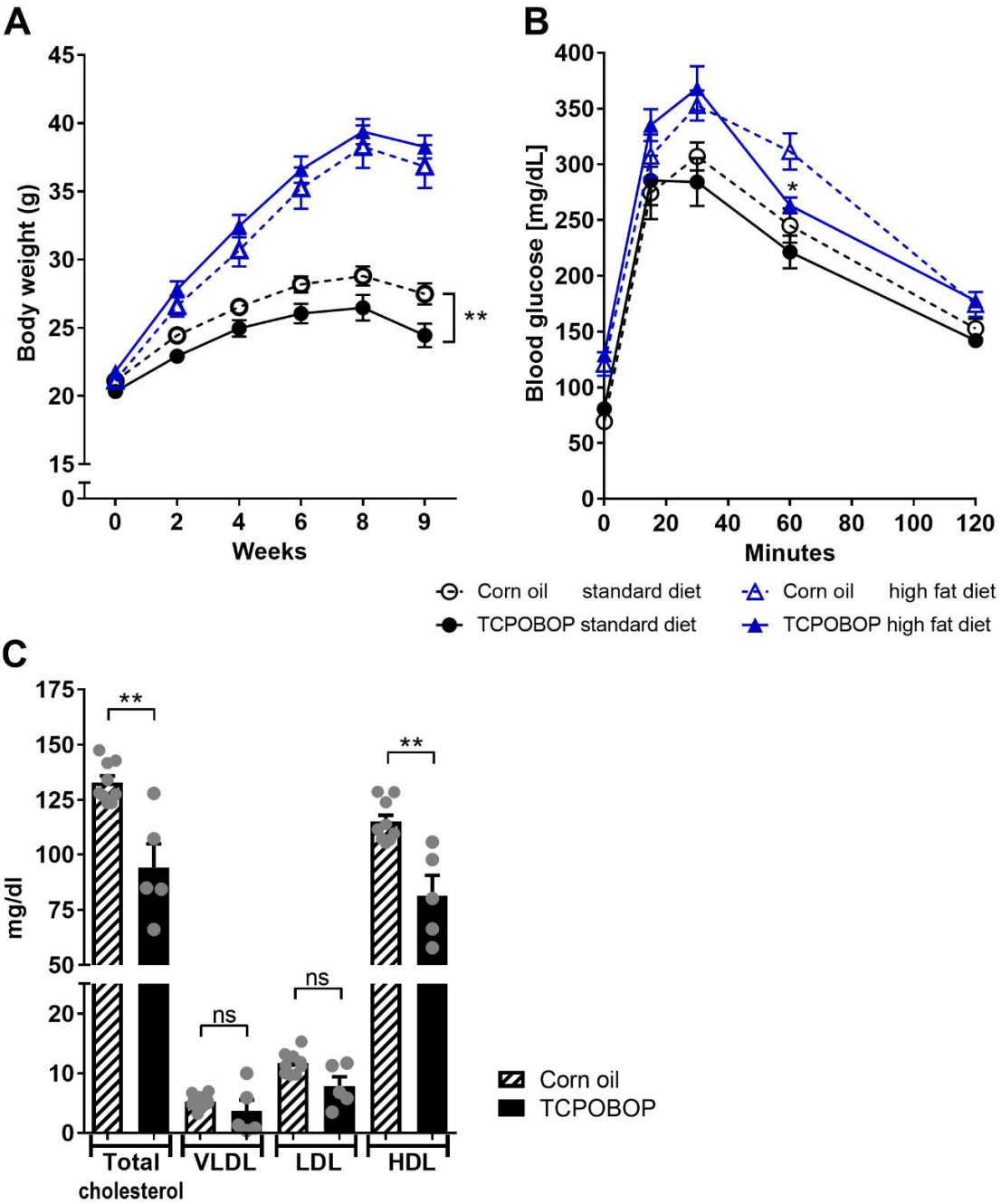
DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 2



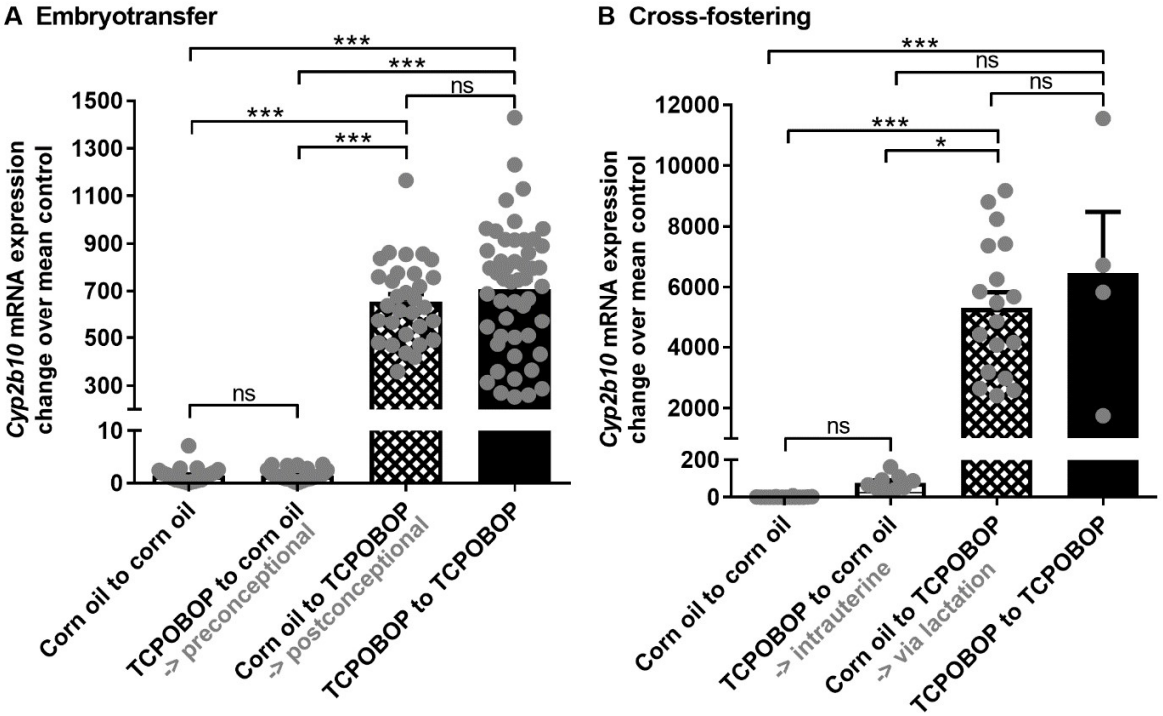
DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3



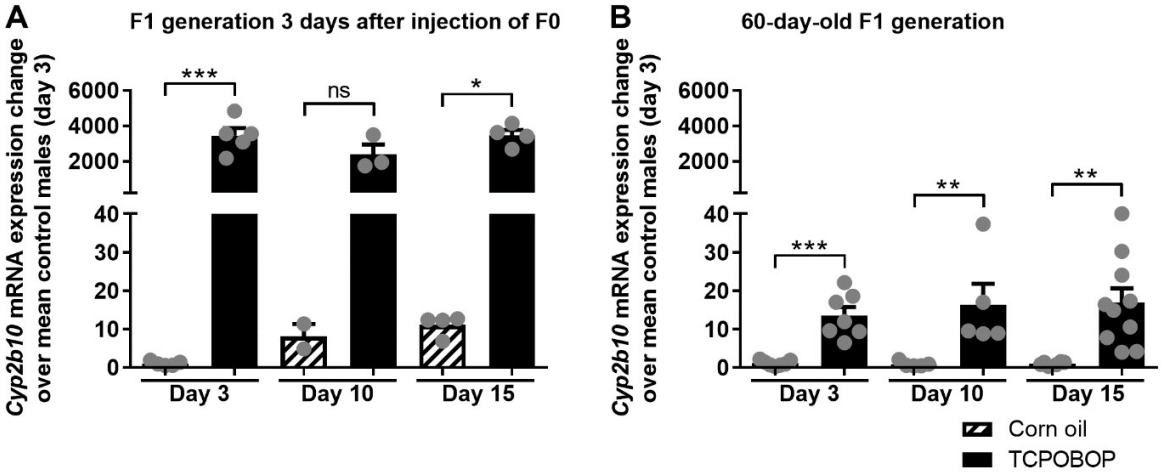
DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4



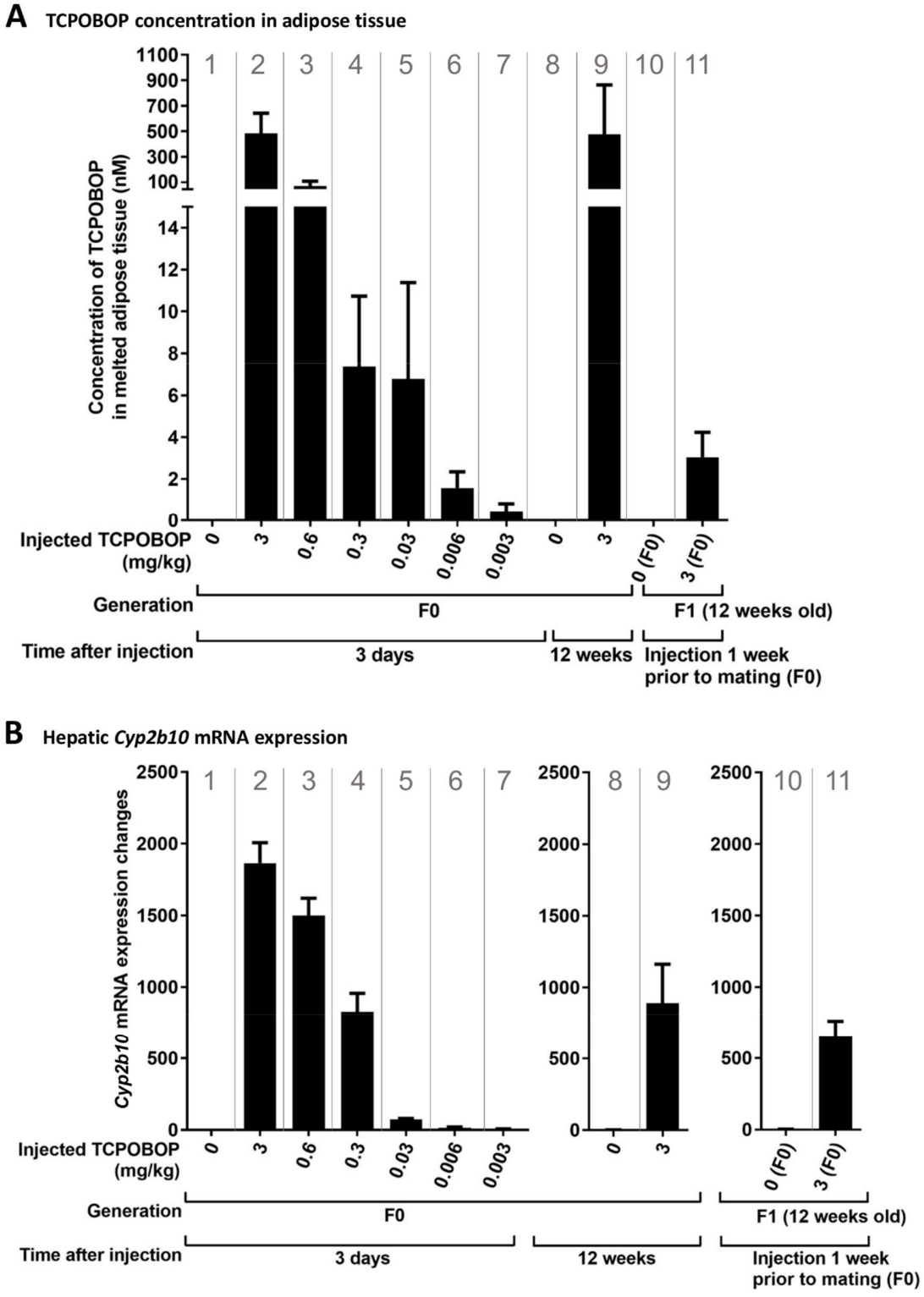
DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 5



DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 6



DMD Fast Forward. Published on August 28, 2018 as DOI: 10.1124/dmd.118.082925
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 7