DMD Fast Forward. Published on November 17, 2015 as DOI: 10.1124/dmd.115.066639
This article has not been copyedited and formatted. The final version may differ from this version.

Age-specific Regulation of Drug-processing Genes in Mouse Liver by Ligands of Xenobiotic-sensing Transcription Factors

Cindy Yanfei Li, Helen J. Renaud, Curtis D. Klaassen and Julia Yue Cui

Department of Environmental & Occupational Health Sciences, University of Washington, Seattle (C.Y.L, C.D.K, J.Y.C); Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kansas (H.J.R)

DMD Fast Forward. Published on November 17, 2015 as DOI: 10.1124/dmd.115.066639
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Developmental Regulation of Drug-processing Genes by Chemicals

Send Correspondence to:

Julia Yue Cui, Ph.D.

Assistant Professor
Department of Environmental & Occupational Health Sciences
School of Public Health
University of Washington
Office: Room. 204
Box: 357234
4225 Roosevelt Way NE
Seattle, WA 98105
Tel: 206-616-4331
Email: juliacui@uw.edu

Text pages: 36

Tables: 4

Figures: 8

References: 47

Abstract: 244 words

Introduction: 715 words

Discussion: 1423 words

Non-Standard Abbreviations:

Abcc (Mrp), ATP-binding cassette; AhR, aryl hydrocarbon receptor; Aldh, aldehyde dehydrogenase; CAR, constitutive androstane receptor; Cyp, cytochrome P450; DPGs, drug-processing genes; Gst, glutathione *S*-transferase; Oatp, organic anion transporter; Papss, 3'-phosphoadenosine 5'-phosphosulfate synthase; PXR, pregnane X receptor; Sult, sulfotransferase; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; Ugt, UDP glucuronosyltransferase; TCPOBOP, 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene; PCN, Pregnane-16α-carbonitrile

ABSTRACT

The xenobiotic-sensing transcription factors (xeno-sensors) aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR) up-regulate the expression of many drug-processing genes (DPGs) in liver. Previous studies have unveiled profound changes in the basal expression of DPGs during development; however, the knowledge of the ontogeny of the inducibility of DPGs in response to pharmacological activation of xeno-sensors is still limited. The goal of this study was to investigate the age-specific regulation of DPGs by prototypical xeno-sensor ligands (TCDD for AhR, TCPOBOP for CAR, and PCN for PXR) during mouse liver development. The basal mRNAs of most DPGs were low during neonatal age, but gradually increased to adult levels, whereas some DPGs (Cyp1a2, Cyp2b10, Cyp3a11, Gstm2, Gstm3, Papss2, and Oatp1a4) exhibited adolescent-predominant expression pattern. The inducibility of DPGs was age-specific: 1) during neonatal age, the highest fold-increase in the mRNA expression was observed for Cyp1a2, Sult5a1 and Ugt1a9 by TCDD; Cyp3a11 and Mrp2 by TCPOBOP; as well as Gstm2 and Gstm3 by PCN; 2) during adolescent age, the highest fold-increase in the mRNA expression was observed for Ugt1a6 and Mrp4 by TCDD; Cyp2b10, Ugt2b34, and Ugt2b35 by TCPOBOP; as well as Gsta1, Gsta4, Sult1e1, Ugt1a1, Mrp3, and Mrp4 by PCN; 3) in adults, the highest fold-increase in the mRNA expression was observed for Aldh1a1, Aldh1a7, and Ugt2b36 by TCPOBOP; as well as Papss2 and Oatp1a4 by PCN. In conclusion, the inducibility of hepatic DPGs following the pharmacological activation of xeno-sensors is age-specific.

INTRODUCTION

Drug-metabolizing enzymes and transporters play crucial roles in the absorption, metabolism, disposition, and elimination of various drugs and other xenobiotics. In liver, which is the major organ for drug metabolism, the drug-metabolizing enzymes are categorized into Phase-I and Phase-II enzymes. Phase-I enzymes are important for the oxidation, reduction, and hydrolysis of xenobiotics. The metabolites of Phase-I reactions can be further metabolized via conjugation reactions by Phase-II enzymes, resulting in increased water solubility of the metabolites, which are ready for elimination. The Phase-II reactions mainly include glucuronidation (which is catalyzed by UDP-glucuronosyl transferases [Ugts]), sulfation (which is catalyzed by sulfotransferases [Sults]), glutathione conjugation (which is catalyzed by glutathione *S*-transferases [Gsts]), as well as other conjugation reactions, such as methylation and amino acid conjugation (Handschin and Meyer, 2003; Wang et al., 2014). Either the parent compounds or their metabolites can be absorbed or eliminated from cells via transporter proteins (Xu et al., 2005; Aleksunes and Klaassen, 2012). The regulation of drug-metabolizing enzymes and transporters (together called Drug-Processing Genes [DPGs]) is important for the pharmacokinetics of drugs, and contribute to adverse drug-drug reactions.

In adults, it is well known that exposure to many xenobiotic chemicals can up-regulate the expression of many Phase-I and -II drug metabolizing enzymes as well as transporters (Xu et al., 2005). Three major xenobiotic-sensing transcription factors, namely aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR), are known to be involved in the regulation of DPGs in adult mouse liver (Aleksunes and Klaassen, 2012).

The AhR is localized in the cytoplasm during its inactive state, but once it is activated by its prototypical ligand TCDD (2,3,7,8-tetrachlorodibenzodioxin), AhR translocates into the nucleus and forms a heterodimer with the AhR nuclear translocator protein (Arnt), which subsequently binds to the xenobiotic response element (XRE) within the cytochrome P450 (Cyp) 1a promoter region, activating the transcription of CYP1a genes (Nakajima et al., 2003; Klaassen and Slitt, 2005). TCDD does not induce Cyp1a mRNA in livers of AhR-null mice (Gonzalez and Fernandez-Salguero, 1998).

Following ligand activation, CAR (activated by the prototypical ligand TCPOBOP) and PXR (activated by the prototypical ligand PCN) both heterodimerize with the Retinoid-X-Receptor (RXR), and up-regulate their prototypical target genes Cyp2b10 and Cyp3a11, respectively (Xu et al., 2005). TCPOBOP-treatment does not increase Cyp2b10 mRNA in CAR-null mice (Wei et al., 2000), and similarly, PXR-null mouse livers had no increase in Cyp3a11 mRNA following PCN-treatment (Aleksunes and Klaassen 2012). Activation of CAR and PXR are important for the up-regulation of many DPGs, and these nuclear receptors are implicated in many adverse drug reactions (Staudinger et al., 2001a; Staudinger et al., 2001b; Hernandez et al., 2009).

Changes in the expression of DPGs during development are associated with differences in drug metabolism between children and adults. Fetal liver microsomes have extremely high CYP3A7 levels, but low CYP3A4, which is the major adult form (Stevens et al., 2003; Stevens et al., 2008). A study of age-dependent CYP2B6 expression also found lower

levels in infant liver when compared to adults (Croom et al., 2009). Because CYPs are not mature at birth, and the mRNA of many hepatic transporters in all pediatric ages is lower than in adults (Anderson, 2002; Mooij et al., 2014), newborns and children are considered more vulnerable to xenobiotic insults (Kearns et al., 2003).

Although we and others have previously characterized the basal ontogenic expression patterns of various DPGs in liver (Hart et al., 2009; Cui et al., 2012a; Cui et al., 2012b; Lu, 2013; Peng, 2013), the knowledge of the inducibility of DPGs in response to pharmacological activation of different xeno-sensors during development is still limited. It is critical to obtain this information because newborns and children are exposed to xenobiotics from the diet and other sources. Although it is known that the DPGs are expressed at various levels during development, it is not known whether these DPGs at younger ages are more or less inducible. Therefore, the goal of this study was to investigate age-specific regulation of DPGs in mouse livers by ligands of xenobiotic-sensing transcription factors, at various ages, namely 2-, 5-, 25-, and 60-days of age. The profiles of gene regulation by three different transcription factors (AhR, CAR and PXR) can potentially provide insights to understand the molecular basis of drug-drug interactions and adverse drug reactions in therapy of children.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA) to be used as breeders. All mice were housed according to the

American Animal Association for Laboratory Animal Care guidelines and were bred under standard conditions at the University of Kansas Medical Center. Mice were allowed *ad libitum* access to food and water and were acclimated to the housing facility for at least 1 week before breeding. The offspring of mice were injected intraperitoneally with a single dose of vehicle (corn oil), AhR ligand (TCDD at 40 µg/kg), CAR ligand (TCPOBOP at 300 µg/kg), or PXR ligand (PCN at 200 mg/kg) at 2-, 5-, 25-, and 60-days of age, and tissues were collected 24h later. Livers were frozen immediately in liquid nitrogen and stored at -80°C. All studies were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Total RNA isolation

Total RNA was isolated from frozen liver tissue using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. The concentration of total RNA was quantified spectrophotometrically at 260 nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The integrity of each RNA sample was evaluated by formaldehyde agarose gel electrophoresis before analysis, and confirmed by visualization of 18S and 28S rRNA bands.

Quantification of mRNA by RT-qPCR assay

Total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA) in a final volume of 10 µl containing 5X iScript reaction mix, 0.5 ul iScript reverse transcriptase, 1 µg total RNA and nuclear-free water. The samples were incubated at 25°C for 5 min, 42°C for 30 min, and reverse transcriptase was inactivated

by heating at 85°C for 5 min. The cDNA samples were subsequently amplified by PCR, using SsoAdvanced Universal SYBR Green Supermix in a Bio-Rad CFX384 Real-Time Detection System (Hercules, CA). A volume of 2 µl of appropriate diluted cDNA sample was added to 8 µl of the PCR master mix, containing SsoAdvanced universal SYBR Green supermix (2X), 200n forward and reverse primer and nuclear-free water. The thermal cycling conditions comprised an initial denature step at 95°C for 30 s, 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The primers for all real-time PCR reactions were purchased from Integrated DNA Technologies (Coralville, IA), and the sequences are shown in Supplement Table 1. The dCq values of each target gene were calculated after subtracting the average Cq values of β-actin, and the ddCq values for each target gene were expressed as percentage of the housekeeping gene β-actin. The full name of each gene and its function are shown in Supplement Table 2.

Western blotting

Whole liver homogenates were prepared using ST buffer (250 mM sucrose, 10 mM tris base, pH 7.5) with protease inhibitor, and protein concentrations were determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. The crude membranes were prepared from these samples as described previously (Aleksunes et al. 2006). The samples were subjected to polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in phosphate-buffered saline with 2.5% Tween 20 (PBST) with 5% nonfat dry milk for 1 h and incubated overnight with one of the following primary antibodies diluted in PBST with 1% milk: 1) rabbit anti-mouse

Cyp2b10 mAb (AB9916, 1:5000, EMD Millipore); 2) mouse anti-rat Cyp3a11 mAb (clone 2-12-1, 1:500), which was a generous gift from Dr. Frank Gonzalez at the National Cancer Institute; 3) rabbit anti-mouse Oatp1a4 (1:1000, University of Kansas Medical Center); and 4) rat anti-mouse Mrp4 (1:2000, Abcam, Cambridge, MA). After washing, the membranes were incubated for 1 h with a species-appropriate secondary antibody (Sigma Aldrich, St. Louis, MO) diluted in PBST with 1% milk. HRP-linked secondary antibodies were applied at 1:2000 to detect the proteins. The membranes were washed and incubated with Novex ECL Chemiluminescent Substrate Reagent Kit (Life Technologies, Carlsbad, CA). Membranes were stripped and re-probed with antibodies against β-actin (Abcam, Cambridge, MA) as the loading control. Intensities of the protein bands were quantified using the Image J Software (National Institutes of Health, Bethesda, MD).

Enzyme Activity in Mouse Liver Microsomes

Liver microsomes were isolated using differential centrifugation as described previously (Pelkonen et al., 1974). Frozen liver samples were weighed and transferred to a Teflon pestle/glass homogenizer with 5 volumes (w/v) ST buffer (10 mM Tris base, 250 mM sucrose, pH 7.5) containing protease inhibitor cocktail (1:100). The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatants were transferred to a clean centrifuge tube and centrifuged at 100,000 g for 60 min at 4°C. The pellets were washed with 1 ml ST buffer, and dissolved in 100 µl of ST buffer with protease inhibitor. P450 enzyme activity was measured using P450-Glo™ Screening system (Promega, Madison, WI). P450 reactions (50 µl) were performed in white opaque 96-well plates. Briefly, a

luminogenic P450-Glo™ substrate was incubated at 37°C with 10 µg of liver microsomal protein, control membrane, or positive P450 enzyme for 10 min (100 µM Luciferin-ME for Cyp1a, 3 µM Luciferin-2B6 for Cyp2b, or 3 µM Luciferin-IPA for Cyp3a). The reactions were initiated by adding the NADPH-regeneration system and incubated for 10 min. At the end of the incubations, an equal volume of the luciferin detection reagent (50 µl) was added at room temperature, and luminescence was quantified 20 min later using a Glomax™ 96 Microplate Luminometer (Promega Corporation, Madison, WI). The magnitude of the light signal is dependent on and directly proportional to the amount of luciferin product generated by the P450 reaction.

Statistical Analysis

Data are presented as mean ± SEM. To test the effect of both age and chemicals on the gene expression of DPGs, data were analyzed using a generalized linear model followed by the Duncan's Post Hoc test ($p<0.05$) using the SPSS software (Armonk, New York). To test the effect of age on the basal mRNA expression of DPGs, data from only corn oil-treated group at various ages were analyzed. Among the DPGs that displayed significant age vs. chemical interactions, individual generalized linear model was applied to identify the inducibility of DPGs by the xenobiotic-sensor ligands at each specific age. Asterisks (*) represent significant differences between vehicle and chemical-treated mice at the same age.

RESULTS

Effect of age and chemicals on the gene expression of DPGs

According to the generalized linear model analysis, 24 out of 29 DPGs displayed statistically significant interaction between age and chemicals, suggesting that the effect of chemical-mediated changes in the mRNA expression of the majority of DPGs was age-specific (Supplement Table.3). Regarding the age effect, the basal mRNAs of most DPGs were lowly expressed during neonatal ages (Day 2 and Day 5), but gradually increased to adult levels; whereas some DPGs (Cyp1a2, Cyp2b10, Cyp3a11, Gstm2, Gstm3, Papss2, Oatp1a4) exhibited an adolescent-predominant expression pattern, with the highest constitutive expression observed at Day 25. Sult1e1 appeared to be the only DPG of which the mRNA was not influenced by age. In summary, age not only impacts the constitutive expression but also the inducibility of DPGs in the liver. The DPGs in each category are described as followings.

Developmental regulation of mRNA for Phase-I drug-metabolizing enzymes by xeno-sensor ligands

To determine the effect of the ligands for the 3 xenobiotic-sensing transcription factors on the expression of their prototypical target genes in liver at various developmental ages, the mRNAs of Phase-I drug-metabolizing enzymes Cyp1a2 (AhR-target), Cyp2b10 (CAR-target), and Cyp3a11 (PXR-target) were quantified in vehicle and ligand-treated groups (Fig. 1A, 1B, 1C). Only the AhR-ligand TCDD markedly up-regulated the mRNA of Cyp1a2 at all ages, and as anticipated neither the CAR-ligand TCPOBOP nor the PXR-ligand PCN altered the Cyp1a2 mRNA expression at any age (Fig. 1A1). Regarding the abundance of Cyp1a2 mRNA, the basal mRNA expression of Cyp1a2 was low at 2-day of age (neonatal) but significantly increased 7.7-fold in 25-day-old mice, and 19.7-fold in

60-day-old mice (Fig. 1A1). Interestingly, compared to the vehicle-treated group of the same age, the fold-induction of Cyp1a2 mRNA was most prominent at 2- and 5-days of age (25.4-fold and 25.2-fold, respectively), compared to 3.3-fold at 25-days of age and 14.9-fold at 60-days of age (Fig. 1A2). Although the age-specific fold-change mediated by TCDD was not the highest in the 60-day adult group, the mRNA abundance of Cyp1a2 in liver of TCDD-treated mice was the highest at this age (Fig. 1A1, 1A2).

Regarding the prototypical CAR-target gene Cyp2b10, only TCPOBOP produced a marked increase in mRNA at all 4 ages as anticipated, and the other 2 chemicals did not alter Cyp2b10 mRNA in liver during development (Fig. 1B1). Regarding the abundance of Cyp2b10 mRNA, the basal mRNA of Cyp2b10 was low at all ages, but significantly increased at Day 25 and Day 60, and the basal Cyp2b10 mRNA was highest at 25-days of age (Fig. 1B1). Compared to the vehicle-treated group of the same age, the fold-induction of Cyp2b10 mRNA was 55.0-fold at 2-days of age, followed by a 279-fold at 5-days of age, which then reached a 400-fold at 25-days of age, and 263-fold at 60-days of age (Fig. 1B2). Therefore, the fold-increase of Cyp2b10 mRNA was up-regulated the most at 25-days of age (adolescent).

The basal expression of Cyp3a11 mRNA was low in neonatal ages, but gradually increased at 25-day of age, and finally reached its adult level by 60-day of age (Fig. 1C1). Compared to the vehicle-treated group of the same age, the AhR-ligand TCDD down-regulated Cyp3a11 mRNA at 2-day (63.1%) and 25-day (57.4%) of age (Fig. 1C2). In contrast, the CAR-ligand TCPOBOP increased Cyp3a11 mRNA at all 4 developmental

ages: 70% at Day 2, 220% at Day 5 (highest fold-increase), 120% at Day 25, and 130% at Day 60. The PXR-ligand PCN-treated groups increased Cyp3a11 mRNA at 5- and 60-days of age (130% and 160%, respectively), but not at the other two ages (Fig. 1C2).

In summary, the expression of the 3 prototypical xeno-sensor target genes has demonstrated that 1) the fold-induction of the xeno-sensor target gene expression is age-specific; 2) there appears to be interactions between different pathways, that is, the AhR-ligand decreases Cyp3a11, and both the CAR- and PXR-ligands can activate the PXR-target gene Cyp3a11. To further investigate the age-specific effects of the 3 xeno-sensor ligands on the expression of drug-metabolism and transport pathways in liver, the mRNA expression of other important drug-processing genes was quantified at the 4 developmental ages. These genes were selected based on a previous publication showing that they are *bona fide* targets of AhR, CAR, and/or PXR in adult male livers (Aleksunes and Klaassen, 2012).

The aldehyde dehydrogenases are important Phase-I enzymes that metabolize aldehydes, and among which, Aldh1a1 and Aldh1a7 are up-regulated by TCPOBOP in adult mouse liver (Aleksunes and Klaassen, 2012). Regarding the mRNA abundance of these Aldhs, all three genes were minimally expressed at 2- and 5-days of age, followed by a gradual increase during development, and the highest mRNA levels in livers were observed in 60-day adult liver (Fig. 1D1, 1E1, 1F1). Compared to the vehicle-treated group of the same age, TCDD did not appear to alter the mRNAs of these Aldhs, except for a 31.8% decrease in the Aldh1a1 at 25-days of age (Fig. 1D2, 1E2, 1F2). In contrast,

consistent with the literature (Aleksunes and Klaassen, 2012), TCPOBOP increased the mRNAs of Aldh1a1 (180%) and Aldh1a7 (150%) in mice at 60-day of age (adult). In addition, TCPOBOP increased the mRNAs of Aldh1a1 (40%) and Aldh1a7 (30%) at 25-day of age, but to a lesser extent. TCPOBOP also increased Aldh1a7 mRNA (100%) in livers of mice at 5-day of age, but it did not alter Aldh1a1 at 5-day of age, or any of the Aldhs in mice at 2-day of age. PCN increased the mRNAs of Aldh1a1 (90%) and Aldh1a7 (60%) at 60-day of age, but decreased Aldh1a1 (65% at Day 5, 68.6% at Day 25) and Aldh1a7 (68% at Day 25) during neonatal and adolescent ages, as well as Aldh7a1 at 5-day of age (35.4%) (Fig. 1D2, 1E2, 1F2).

Developmental regulation of Phase-II glutathione *S*-transferases (Gsts) and Sulfotransferases (Sults) by xeno-sensor ligands

The mRNA expression of Gst isoforms was minimally expressed at 2- and 5-days of age (neonatal), but gradually increased to adult levels by 25- or 60-days of age (Fig. 2A1-2G1). Compared to the vehicle-treated group of the same age, the PXR-ligand PCN increased the mRNA expression of Gsta1 (110%) and Gsta4 (40%) at 25-day of age (Fig. 2A2 and 2B2) and the AhR-ligand TCDD only increased the mRNA of Gsta1 at 60-day of age (63.5%), whereas none of the three chemicals significantly altered the mRNAs of Gstm1 and Gstm4 (Fig. 2C2, 2F2). Regarding the other Gstm isoforms, the PXR-ligand PCN up-regulated Gstm2 mRNA (48%) at 2-day of age, and Gstm3 mRNA at all ages (170% at Day 5, 80% at Day 25, 2.4-fold at Day 60, respectively) (Fig. 2D2 and 2E2). The AhR-ligand TCDD induced Gstm2 mRNA (23%) at 60-days of age (Fig. 2D2, 2E2). In contrast, PCN appeared to down-regulate Gstt1 mRNA (23%) at 5-day of age (Fig. 2G2).

As for the expression of Sult isoforms in liver, Sult1e1 mRNA was lowly expressed at all ages (Fig 3A1) with no statistical difference. Compared to the vehicle-treated group of the same age, the mRNA expression of Sult1e1 was highly inducible by PCN at 25-day of age (10.1-fold) (Fig. 3A2). Regarding the abundance of Sult5a1 mRNA, it gradually increased from neonatal level and finally reached its adult level at 60-day of age. Compared to the vehicle-treated group of the same age, Sult5a1 mRNA was induced by TCDD at both 5-day (4.3-fold) and 60-day (120%) of age, and the highest fold-increase of Sult5a1 was observed in 5-day-old mice. The Sult5a1 mRNA was also up-regulated by TCPOBOP at 60-day of age (100%), but down-regulated by PCN at 25-day of age (79% decrease) (Fig. 3B2). With regard to the enzyme that synthesizes the co-substrate for sulfation reactions, namely 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (Papss2), its basal mRNA was minimally expressed at neonatal ages, but gradually increased to adult level, and the highest basal mRNA expression of Papss2 was observed in liver of 25-day-old mice (Fig. 3C1). Compared to the vehicle-treated group of the same age, TCDD decreased the Papss2 mRNA at Day 2 (50%) and Day 25 (56%), but not at the other ages (Fig. 3C2). TCPOBOP up-regulated Papss2 mRNA at all ages except for 2-days of age (80% at Day 5, 60% at Day 25 and 100% at Day 60, respectively). PCN up-regulated Papss2 mRNA only at 60-day of age (120%), but moderately decreased Papss2 mRNA at 25-day of age (34%) (Fig. 3C2).

Developmental regulation of Phase-II UDP-glucuronosyltransferases (Ugts) by xeno-sensor ligands

To determine the effect of xenobiotic-sensor ligands mediated changes in the expression of Ugts that are known to be *bona fide* targets of at least one of the 3 xenobiotic-sensing transcription factors in adult mice (Aleksunes and Klaassen, 2012), the mRNAs of these Ugt isoforms, namely Ugt1a1, 1a6, 1a9, 2b34, 2b35, and 2b36 were quantified at the 4 developmental ages (Fig. 4A1-4F1). Regarding the basal expression of Ugt1a1 mRNA, it gradually increased to adult levels by 60-day of age (Fig. 4A1). Compared to the vehicle-treated group of the same age, Ugt1a1 mRNA was inducible by the CAR-ligand TCPOBOP at both 2- (70%) and 25-day of age (60%), as well as by the PXR-ligand PCN at 25-day of age (80%). The highest fold-change was observed in livers from 25-day-old, PCN-treated mice (Fig. 4A2). This is consistent with our previous findings that hepatic Ugt1a1 expression was inducible by the CAR-ligand TCPOBOP or the PXR-ligand PCN, but to a lesser extent. The reason for this lower level of induction is probably due to the different dosage schedule, using only a single injection in this study, compared to previous study in which the mice were dosed once daily for four consecutive days (Aleksunes and Klaassen, 2012).

The basal mRNA expression of Ugt1a6 and Ugt1a9 was low in neonatal ages, but gradually increased to adult levels by 60-day of age (Fig 4B1, 4C1). Both Ugt1a6 and Ugt1a9 were up-regulated by the AhR-ligand TCDD in an age-specific manner (Fig. 4B1, 4C1). Compared to the vehicle-treated group of the same age, Ugt1a6 mRNA was inducible by TCDD at 25-day (110%) and 60-day (70%) of age. Although the highest fold-increase of Ugt1a6 was observed in the TCDD-treated group at 25-days of age (Fig. 4B2), the highest mRNA values were seen in 60-day-old TCDD-treated mice (Fig. 4B1). The

fold-induction of Ugt1a9 mRNA by TCDD was observed at both 5-day (40%) and 60-day (40%) of age (Fig. 4C2). Consistently, the highest mRNA value of Ugt1a9 was also observed in 60-day-old TCDD-treated mice (Fig. 4C1).

Ugt2b34 mRNA increased gradually to adult levels by 25-days of age (Fig. 4D1), whereas Ugt2b35 mRNA was minimally expressed at 2- and 5-days of age, followed by significant increase only at 60-day of age (Fig. 4E1). Compared to the vehicle-treated group of the same age, TCDD had no effect on the mRNAs of Ugt2b34 or 2b36 at any age (Fig. 4D1, 4F1), whereas it significantly induced the mRNA expression of Ugt2b35 at 25-day of age (81.2%). TCPOBOP increased the mRNAs of Ugt2b34 at all ages except 2-day of age (50% at Day-5, 150% at Day-25 and 100% at Day-60). In contrast, PCN increased Ugt2b34 only at Day 60 (100%). The highest fold-increase of Ugt2b34 mRNA was seen in 25-day-old TCPOBOP-treated mice (Fig. 4D2). Similarly, TCPOBOP also induced the mRNA of Ugt2b35 at all ages except at 5-day of age (100% at Day 2, 120% at Day 25, and 110% at Day 60). As shown, the highest fold-increase of Ugt2b35 was seen in 25-day-old TCPOBOP-treated mice (Fig. 4E2). The fold-increase of Ugt2b36 mRNA was only seen in 60-day-old TCPOBOP-treated mice (70%) (Fig. 4F2).

Developmental regulation of transporters by xeno-sensor ligands

The mRNA of the uptake transporter Oatp1a4 was minimally expressed at neonatal ages (2- and 5-day of age), but significantly increased after 25-day of age (Fig. 5A1). Compared to the vehicle-treated group of the same age, the mRNA of Oatp1a4 was significantly down-regulated by TCDD at both Day 2 and Day 25 (61.6% and 62.8%,

respectively), whereas it was up-regulated by TCPOBOP (60%) and PCN (100%) at 5-day of age (Fig. 5A2). At 25-day of age, PCN up-regulated Oatp1a4 mRNA (60%). At 60-day of age, neither TCDD nor TCPOBOP altered the quantity of Oatp1a4 mRNA; however, PCN markedly induced the fold-change of Oatp1a4 mRNA (5.8-fold) (Fig. 5A2).

The mRNA of efflux transporters Mrp2 and Mrp3 were gradually increased to adult levels at 60-day of age (Fig. 5B1, 5C1). Compared to the vehicle-treated group of the same age, the mRNA of Mrp2 was not altered by the AhR-ligand TCDD at any age; however, it was markedly up-regulated by the CAR-ligand TCPOBOP (5.3-fold) and the PXR-ligand PCN (3.7-fold) at 2-days of age. It was also up-regulated by TCPOBOP at Day-25 (80%) and Day-60 (80%), but to a lesser extent (Fig. 5B2). Compared to the vehicle-treated group of the same age, the mRNA of Mrp3 was not altered by any of these three chemicals at 2-day or 60-day of age, but with minor up-regulation by TCDD at Day 25 (50%) (Fig. 5C2). TCPOBOP also up-regulated Mrp3 mRNA at 5-day (90%) and 25-day (80%) of age. The highest fold-increase of Mrp3 mRNA was observed in 25-day-old PCN-treated mice (140%) (Fig. 5C2). Comparing to the vehicle-treated group of the same age, Mrp4 mRNA was inducible by all these 3 chemicals at various ages. The CAR-ligand, TCPOBOP, up-regulated Mrp4 mRNA at both 2-day (50%) and 5-day (80%) of age, but not at the other two ages. The AhR-ligand, TCDD, induced a fold-increase of Mrp4 mRNA at both 25-day (190%) and 60-day (130%) of age. As shown, the highest fold-increase of Mrp4 mRNA was observed in 25-day-old PCN-treated mice (3.8-fold) (Fig. 5D2).

In summary, as shown in Table 1, some of the DPGs were most induced at the neonatal ages (2- and 5-days of age), whereas others were induced most at the adolescent or adult ages. For example, the DPGs were up-regulated in livers of neonatal mice with the highest fold induction observed for the mRNAs of Cyp1a2, Sult5a1 and Ugt1a9 by TCDD; Cyp3a11 and Mrp2 by TCPOBOP; Gstm2 and Gstm3 by PCN. Some DPGs were up-regulated the most in livers of adolescent mice with the highest fold-increase observed for the mRNAs of Ugt1a6 and Mrp4 by TCDD; Cyp2b10, Ugt2b34, and Ugt2b35 by TCPOBOP; as well as Gsta1, Gsta4, Sult1e1, Ugt1a1, Mrp3, and Mrp4 by PCN. Other DPGs were up-regulated the most in adult livers: Aldh1a1, Aldh1a7 and Ugt2b36 by TCPOBOP; as well as Papss2, and Oatp1a4 by PCN. Therefore, a marked difference in the fold-induction of the prototypical xeno-sensor target genes was observed during liver development, suggesting that age plays a major role in the sensitivity of the hepatic drug-processing machinery to xenobiotics.

Expression of xenobiotic-sensing transcription factors

To determine whether the chemicals that activate the 3 transcription factors also increase the mRNA of the transcription factors, the mRNAs of AhR, CAR, and PXR were quantified in control and the ligand-treated conditions. Regarding the abundance of AhR, CAR, and PXR mRNA, they were minimally expressed at Day-2 and Day-5 neonatal ages, but reached the highest mRNA level at the adolescent age of Day-25 (Fig. 6A1, 6B1, 6C1). Compared to the vehicle-treated group, the AhR mRNA was not altered by any chemical at Day-2 or Day-5 neonatal ages; however, at Day-25, the AhR-ligand TCDD and the PXR-ligand PCN both decreased the AhR mRNA 46.5% and 67.7%, respectively. At 60-

days of age, both the CAR ligand TCPOBOP and PXR-ligand PCN up-regulated the AhR mRNA (150% and 200%, respectively) (Fig. 6A2)

The basal mRNA expression of the transcription factor CAR was significantly increased in both 25- and 60-day-old mice as compared to its neonatal level (Fig. 6B1). Compared with the vehicle-treated group of the same age, the mRNA of CAR was not altered by its ligand TCPOBOP at any age. However, at Day 25 and Day 60, the AhR-ligand TCDD induced the mRNA expression of CAR (90% and 180% respectively); whereas this effect was not observed during neonatal ages. Interestingly, the PXR-ligand PCN also up-regulated CAR mRNA at 2-days of age (100%). But during development, PCN down-regulated CAR mRNA at both Day 5 (48.6%) and Day 25 (63.5%). The highest fold-change of CAR mRNA was observed in 60-day-old TCDD-treated mice (Fig. 6B2).

Similarly, the basal mRNA expression of the transcription factor PXR was also significantly expressed after 25-day of age (Fig. 6C1). Compared with the vehicle-treated group of the same age, PXR mRNA was not altered by any of the 3 chemicals in 5- and 60-day-old mice (Fig. 6C2). At 2-day of age, TCDD down-regulated PXR mRNA (64.4%), whereas PCN markedly up-regulated PXR mRNA (150%, highest fold change). At 25-days of age, both TCDD and PCN decreased PXR mRNA (30.1% and 48.1%, respectively) (Fig. 6C2).

Protein expression

Western blot analysis was conducted for the Phase-I drug-metabolizing enzymes Cyp2b10, Cyp3a11, uptake transporter Oatp1a4, and efflux transporter Mrp4 (Fig. 7A). Compared to the vehicle-treated group at the same age, Cyp2b10 protein was persistently up-regulated by CAR-ligand TCPOBOP at all ages (3.5-fold, 5.9-fold and 5.1-fold, respectively), whereas the PXR-ligand PCN induced 4.1-fold increase of Cyp2b10 protein only at 25-days of age. At 5- and 60-days of age, PCN tended to increase 87% and 32% of Cyp2b10 protein expression although a statistically significance was not achieved (Fig. 7E). Another drug-metabolizing enzyme Cyp3a11 was markedly increased in response to TCPOBOP (141%) and PCN (115%) at 5-days of age. Cyp3a11 protein was also induced by both TCPOBOP (40%) and PCN (65.3%) at 25-days of age but to a lesser extent. However, the protein expression of Cyp3a11 was not altered by any of these 3 chemicals in adult mice (Fig. 7D), suggesting the possibility of post-transcriptional control during development. The protein expression of Oatp1a4 was not altered by TCPOBOP at any age group, but in PCN-treated mice Oatp1a4 was elevated at both 25- and 60-days of age (30.6% and 67%, respectively) (Fig. 7C). The protein expression of Mrp4 was increased by both TCPOBOP (60.9%) and PCN (78.5%) at 5-days of age. At 25-days of age, PCN also increased Mrp4 protein expression (69.1%) (Fig. 7B)

Enzyme activities

To determine the enzyme activities of target genes of xenobiotic-sensing transcription factors, three major DPGs, namely Cyp1a, Cyp2b, and Cyp3a, were quantified (Fig. 8A, 8B, 8C). Because of the limited amount of mouse livers collected at 2-days age, the

enzyme activities were only conducted at 5-, 25- and 60-days age groups. Compared to the vehicle-treated group of the same age, the fold-induction of Cyp1a activity was most prominent at the 5-days of age (3.5-fold), followed by a moderate up-regulation at 25- and 60-days of age (40% and 20%, respectively) (Fig. 8A). The activity of Cyp2b was significantly up-regulated by TCPOBOP at 25- and 60-days of age compared with the activity in the vehicle-treated groups (140% and 90%, respectively), with the highest fold induction in 25-day-old adolescent mice (Fig. 8B). The Cyp3a activity was induced in TCPOBOP-treated mouse livers (2.8-fold, 6-fold, and 8.7-fold respectively) at all ages, whereas this induction was also observed in PCN-treated group only at 25- and 60-days of age (18.4-fold and 17-fold, respectively) (Fig. 8C).

Discussion

In conclusion, the current study has provided critical information regarding the age-specific regulation of drug-metabolizing enzymes and transporters in mouse liver by the prototypical ligands of three major xenobiotic-sensing transcription factors, including AhR-ligand TCDD, CAR-ligand TCPOBOP, and PXR-ligand PCN. This study has demonstrated that age not only influences the constitutive expression but also the inducibility of DPGs in liver by these chemicals.

Characterization of the developmental expression patterns of DPGs is of importance to predict drug clearance, efficacy, and risk of adverse drug reactions in neonates and children (Hines, 2007). In adults, it is well known that exposure to microsomal enzyme inducers can induce the expression of some Phase-I and Phase-II drug metabolizing

enzymes as well as transporters (Aleksunes and Klaassen, 2012). Earlier studies have characterized the ontogeny of many drug metabolizing enzymes during human liver development (de Wildt et al., 1999), including hepatic cytochrome P450s (Hines, 2007), flavin-containing monooxygenases (FMO) (Hines, 2006), sulfotransferases (Richard et al., 2001), UDP-glucuronyltransferases (Strassburg et al., 2002) as well as transporters (Mooij et al., 2014). There are profound changes occurring in the basal ontogenic expression of drug-metabolizing enzymes in human liver during development.

Little is known about the inducibility of DPGs in pediatric patients, mainly due to ethical concerns for conducting chemical exposure experiments in humans during development. Considering the highly conserved nature of numerous DPGs between mouse and humans, the mouse model has become a common tool to recapitulate the regulation of the DPGs in humans. Moreover, the mouse model has many technical advantages such as easy to maintain, minimized influences from gene variation, environment, and dietary factors. Lots of studies using wild-type, knockout, and transgenic mice have reported gene expression profiles in mouse livers for many DPGs (Cui et al., 2009; Hart et al., 2009; Aleksunes and Klaassen, 2012), and these studies have provided mechanistic information into the regulation of DPGs *in vivo*.

In mouse livers, using RNA-sequencing, we have characterized the ontogenic expression of many drug-processing genes under basal conditions at various ages, including various Phase-I enzymes (Peng et al., 2012; Peng et al., 2013), Phase-II enzymes (Lu et al., 2013) and transporters (Cui et al., 2012a). However, there is a critical gap in the literature

regarding whether xenobiotics that activate AhR, CAR, and PXR are able to increase the expressions of DPGs in younger animals as in adults. Previous studies have illustrated various inducibility of drug metabolizing enzymes during development in different species. For example, the hepatic glutathione *S*-transferase (Gst) exhibited functional heterogeneity in mice, rats, rabbits and guinea pigs after pretreatment with Gst inducers (Gregus et al., 1985); The ontogenetic expression and localization of Cyp1a1 and Cyp1a2 in the livers of rabbits are differently regulated with increasing age (Rich et al., 1993); More studies used the rat model to identify the activities of P450 after maximal induction, such as Cyp1a1/1a2 and 2b1/2b2 by phenobarbital (Horbach et al., 1992; Agrawal and Shapiro, 1996), Cyp3a by dexamethasone (Lee and Werlin, 1995; Wauthier et al., 2004), or Cyp induction (1a, 2b, 3a) by inducer dimethylcyclosiloxanes in fetal liver of rats (Falany and Li, 2005). However, most of these studies are limited to one category of drug metabolizing enzymes in response to a single inducer. Moreover, it appears that the effect of age cannot be generalized for the various P450s, and the influence of age on one specific P450 is dependent on the type of inducer used (Horbach et al., 1990). Therefore, our study has filled the critical knowledge gap regarding the inducibility of the DPGs at various ages.

Studies have shown that activation of these transcription factors by specific inducers play an important role in the induction of their associated target genes in adult mouse livers (Aleksunes and Klaassen, 2012). As expected, in the present study, the prototypical target genes for each transcription factor were markedly induced in adult mice in response to their corresponding ligands. For example, the AhR-ligand TCDD, CAR-ligand

TCPOBOP, and PXR-ligand PCN markedly up-regulated the mRNAs of Cyp1a2, Cyp2b10, and Cyp3a11, respectively, in adult mouse livers (Fig. 1). Similar to findings in the prior publication, these three xeno-sensor ligands were also involved in the up-regulation of target genes of other transcription factors. For example, previous studies using AhR-null mice reported that Sult5a1, Ugt1a6, and Ugt1a9 were all AhR-target genes, whereas in this study TCDD also induced the mRNA expression of some Phase-II drug-processing genes (such as Sult5a1, Ugt1a6 and Ugt1a9) in liver of 60-day-old mice (Fig. 3B1, 4B1, 4C1) (Petrick and Klaassen, 2007; Aleksunes and Klaassen, 2012). The activation of CAR signaling by TCPOBOP resulted in the up-regulation of a series of genes as shown in Fig.1-5, including Phase-I (Cyp3a11, Aldh1a1, Aldh1a7), Phase-II (Sult5a1, Papss2, Ugt2b34, Ugt2b35, Ugt2b36), and transporters (Mrp2). As illustrated in previous studies, these genes are known CAR-target genes, and significantly induced by TCPOBOP to various degrees, for example Cyp3a11 mRNA in adult male mice was induced 2- to 3-fold, Aldh1a1 2-fold, Aldh1a7 3-fold, and Mrp2 1.5-fold (Aleksunes and Klaassen, 2012). In addition, activation of PXR by its ligand PCN also significantly induced the expression of the Oatp1a4 transporter, as shown in the previous study. The consistent findings of the present study in adult mice livers provide evidence for the involvement of transcription factors in the regulation of Phase-I, Phase-II, and transporter expression.

Some DPGs, which shown to be induced by the transcription factor ligands in the previous literature (Aleksunes and Klaassen, 2012), were not significantly altered in the present study. For example, the prior studies on adult male mice livers showed up-regulation of

the mRNA expressions by AhR-ligand TCDD (Cyp2b10 and Ugt2b35); CAR-ligand TCPOBOP (Cyp1a2, Gsta1, Gsta4, Gstm1-m4, Gstt1, all Sult enzymes, Ugt1a1, Ugt1a9, Mrp3, and Mrp4); and PXR-ligand PCN (Gsta1, Gstm1-m3, Ugt1a1, and Ugt1a9) (Aleksunes and Klaassen, 2012). This difference may be due to the differences in the dosing regimen, in that mice in the present study were treated only once with the same dose as previous studies in which the mice received four consecutive-days of treatment. However, even though these genes were not altered by ligands at 60-days of age in this study, there were remarkable up-regulation occurring at younger ages, suggesting that younger ages are more sensitive to the xenobiotic-mediated DPG up-regulation.

The mRNA and protein up-regulation of Cyp1a2 by TCDD was consistent at 5-days of age; however, we did not observe a significant up-regulation of the Cyp1a2 protein at other developmental ages, whereas the Cyp1a2 mRNA remained to be up-regulated. The mechanisms underlying such discrepancy between the mRNA and the protein levels remain unknown, but it is possible that post-transciptional modifications, such as mRNA degradation by miRNAs, may play a role. These mechanisms will be addressed in future studies. Another examples are that, at 25-days age, PCN tended to increase the Cyp3a11 mRNA, although a statistical significance was not achieved; however, PCN significantly up-regulated the Cyp3a11 protein and enzyme activity at 25-days of age. At 60-days of age, the Cyp3a11 mRNA was up-regulated by both TCPOBOP and PCN, but there was no significant change on the protein level in these two treatment groups. Such discrepancy is likely regulated by post-transcriptional mechanisms.

Although the regulation of many DPGs share great similarities between mice and humans, there are certain species differences in the nuclear receptors mediated gene expression (Gregus et al., 1985; Dickins, 2004; Lin, 2006; Hewitt et al., 2007; Mueller et al., 2010; Gonzalez et al., 2015). For example, TCPOBOP can only activate the mouse CAR resulting in an up-regulation of Cyp2b, but not the human CAR target gene CYP2B6 (Dickins, 2004); PCN is a potent activator of the mouse PXR resulting in an up-regulation of Cyp3a, but it has little effect on the human PXR, which alternatively is known to be induced by rifampicin (Hewitt et al., 2007). Activation of the mouse CAR by phenobarbital results in increased cell proliferation and liver tumor formation; however, phenobarbital does not cause tumor in humans, and in fact, it has long been used as a therapeutic drug to treat convulsion (Whysner et al., 1996). The species differences of the nuclear receptors are likely due to the differences in their ligand binding domains between human and rodents. Therefore, it is important to compare the mouse data with the pediatric patient data in future studies.

Taken together, the present data provide important insight into the age-specific xenobiotic regulation of drug-metabolizing and transporter genes during development. Understanding this gene regulation is critical to predict drug disposition, distribution, metabolism and elimination across all ages, and can thus decrease the risk of an adverse reaction during drug treatment in pediatric patients.

Acknowledgements

The authors would like to thank all laboratory members for the sample preparation, and rotation student Ms. Carly Strecker Wilder for her assistance in the primer design.

Author Contributions:

Participated in research design: Renaud, Klaassen, Cui.

Conducted experiments: Li, Renaud, Cui.

Performed data analysis: Li.

Wrote or contributed to the writing to the manuscript: Li, Renaud, Klaassen, Cui.

DMD Fast Forward. Published on November 17, 2015 as DOI: 10.1124/dmd.115.066639
This article has not been copyedited and formatted. The final version may differ from this version.

Reference

Agrawal AK and Shapiro BH (1996) Phenobarbital induction of hepatic CYP2B1 and CYP2B2: pretranscriptional and post-transcriptional effects of gender, adult age, and phenobarbital dose. *Mol Pharmacol* **49:**523-531.

Aleksunes LM and Klaassen CD (2012) Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARα-, and Nrf2-null mice. *Drug Metab Dispos* **40:**1366-1379.

Anderson GD (2002) Children versus adults: pharmacokinetic and adverse -effect differences. *Epilepsia* **43 Suppl 3:**53-59.

Croom EL, Stevens JC, Hines RN, Wallace AD, and Hodgson E (2009) Human hepatic CYP2B6 developmental expression: the impact of age and genotype. *Biochem Pharmacol* **78:**184-190.

Cui JY, Gunewardena SS, Yoo B, Liu J, Renaud HJ, Lu H, Zhong XB, and Klaassen CD (2012a) RNA-Seq reveals different mRNA abundance of transporters and their alternative transcript isoforms during liver development. *Toxicol Sci* **127:**592-608.

Cui JY, Renaud HJ, and Klaassen CD (2012b) Ontogeny of novel cytochrome P450 gene isoforms during postnatal liver maturation in mice. *Drug Metab Dispos* **40:**1226-1237.

Cui YJ, Cheng X, Weaver YM, and Klaassen CD (2009) Tissue distribution, gender-divergent expression, ontogeny, and chemical induction of multidrug resistance transporter genes (Mdr1a, Mdr1b, Mdr2) in mice. *Drug Metab Dispos* **37:**203-210.

de Wildt SN, Kearns GL, Leeder JS, and van den Anker JN (1999) Cytochrome P450 3A: ontogeny and drug disposition. *Clin Pharmacokinet* **37:**485-505.

Dickins M (2004) Induction of cytochromes P450. *Curr Top Med Chem* **4:**1745-1766.

Falany CN and Li G (2005) Effects of age and pregnancy on cytochrome P450 induction by octamethyltetracyclosiloxane in female Sprague-Dawley rats. *J Biochem Mol Toxicol* **19:**129-138.

Gonzalez FJ, Fang ZZ, and Ma X (2015) Transgenic mice and metabolomics for study of hepatic xenobiotic metabolism and toxicity. *Expert Opin Drug Metab Toxicol* **11:**869-881.

Gonzalez FJ and Fernandez-Salguero P (1998) The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos* **26:**1194-1198.

Gregus Z, Varga F, and Schmelás A (1985) Age-development and inducibility of hepatic glutathione S-transferase activities in mice, rats, rabbits and guinea-pigs. *Comp Biochem Physiol C* **80:**85-90.

Handschin C and Meyer UA (2003) Induction of drug metabolism: the role of nuclear receptors. *Pharmacol Rev* **55:**649-673.

Hart SN, Cui Y, Klaassen CD, and Zhong XB (2009) Three patterns of cytochrome P450 gene expression during liver maturation in mice. *Drug Metab Dispos* **37:**116-121.

Hernandez JP, Mota LC, and Baldwin WS (2009) Activation of CAR and PXR by Dietary, Environmental and Occupational Chemicals Alters Drug Metabolism, Intermediary Metabolism, and Cell Proliferation. *Pharmgenomics Pers Med* **7:**81-105.

Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, and Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39:**159-234.

Hines RN (2006) Developmental and tissue-specific expression of human flavin-containing monooxygenases 1 and 3. *Expert Opinion Drug Metab Toxicol* **2:**41-49.

Hines RN (2007) Ontogeny of human hepatic cytochromes P450. *J Biochem Mol Toxicol* **21:**169-175.

Horbach GJ, Van Asten JG, Rietjens IM, Kremers P, and Van Bezooijen CF (1992) The effect of age on inducibility of various types of rat liver cytochrome P-450. *Xenobiotica* **22:**515-522.

Horbach GJ, Van Asten JG, and Van Bezooijen CF (1990) The influence of age on the inducibility of rat liver cytochrome P450IA1 (CYPIA1) and P450IA2 (CYPIA2) mRNAs. *Mutat Res* **237:**117-121.

Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, and Kauffman RE (2003) Developmental pharmacology--drug disposition, action, and therapy in infants and children. *N Engl J Med* **349:**1157-1167.

Klaassen CD and Slitt AL (2005) Regulation of hepatic transporters by xenobiotic receptors. *Curr Drug Metab* **6:**309-328.

Lee PC and Werlin SL (1995) The induction of hepatic cytochrome P450 3A in rats: effects of age. *Proc Soc Exp Biol Med* **210:**134-139.

Lin JH (2006) CYP induction-mediated drug interactions: in vitro assessment and clinical implications. *Pharm Res* **23:**1089-1116.

Lu H (2013) RNA-Sequencing Quantification of Hepatic Ontogeny and Tissue Distribution of mRNAs of Phase II Enzymes in Mice. *Drug Metab Dispos* **41:**844-857.

Lu H, Gunewardena S, Cui JY, Yoo B, Zhong XB, and Klaassen CD (2013) RNA-sequencing quantification of hepatic ontogeny and tissue distribution of mRNAs of phase II enzymes in mice. *Drug Metab Dispos* **41:**844-857.

Mooij MG, Schwarz UI, de Koning BAE, Leeder JS, Gaedigk R, Samsom JN, Spaans E, van Goudoever JB, Tibboel D, Kim RB, and de Wildt SN (2014) Ontogeny of Human Hepatic and Intestinal Transporter Gene Expression during Childhood: Age Matters, in: *Drug Metab Dispos*, pp 1268-1274.

Mueller SO, Fery Y, Tuschl G, and Schrenk D (2010) Species-specific activation of nuclear receptors correlates with the response of liver drug metabolizing enzymes to EMD 392949 in vitro. *Toxicol Lett* **193:**120-123.

Nakajima M, Iwanari M, and Yokoi T (2003) Effects of histone deacetylation and DNA methylation on the constitutive and TCDD-inducible expressions of the human CYP1 family in MCF-7 and HeLa cells. *Toxicol Lett* **144:**247-256.

Pelkonen O, Kaltiala EH, Larmi TKI, and Kärki NT (1974) Cytochrome P-450-linked monooxygenase system and drug-induced spectral interactions in human liver microsomes. *Chemico-Biological Interactions* **9:**205-216.

Peng L (2013) RNA-sequencing quantification of hepatic ontogeny of phase-I enzymes in mice, in: *Drug Metab Dispos*, pp 2175-2186.

Peng L, Cui JY, Yoo B, Gunewardena SS, Lu H, Klaassen CD, and Zhong XB (2013) RNA-sequencing quantification of hepatic ontogeny of phase-I enzymes in mice. *Drug Metab Dispos* **41:**2175-2186.

Peng L, Yoo B, Gunewardena SS, Lu H, Klaassen CD, and Zhong XB (2012) RNA sequencing reveals dynamic changes of mRNA abundance of cytochromes P450 and their alternative transcripts during mouse liver development. *Drug Metab Dispos* **40:**1198-1209.

Petrick JS and Klaassen CD (2007) Importance of hepatic induction of constitutive androstane receptor and other transcription factors that regulate xenobiotic metabolism and transport. *Drug Metab Dispos* **35:**1806-1815.

Rich KJ, Foster JR, Edwards RJ, Davies DS, and Boobis AR (1993) Ontogenetic development of the distribution of constitutive and 3-methylcholanthrene-induced CYP1A1 and CYP1A2 in rabbit liver. *J Histochem Cytochem* **41:**915-925.

Richard K, Hume R, Kaptein E, Stanley EL, Visser TJ, and Coughtrie MW (2001) Sulfation of thyroid hormone and dopamine during human development: ontogeny of phenol sulfotransferases and arylsulfatase in liver, lung, and brain. *J Clin Endocrinol Metab* **86:**2734-2742.

Staudinger J, Liu Y, Madan A, Habeebu S, and Klaassen CD (2001a) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* **29:**1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, and Kliewer SA (2001b) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98:**3369-3374.

Stevens JC, Hines RN, Gu C, Koukouritaki SB, Manro JR, Tandler PJ, and Zaya MJ (2003) Developmental expression of the major human hepatic CYP3A enzymes. *J Pharmacol Exp Ther* **307:**573-582.

Stevens JC, Marsh SA, Zaya MJ, Regina KJ, Divakaran K, Le M, and Hines RN (2008) Developmental changes in human liver CYP2D6 expression. *Drug Metab Dispos* **36:**1587-1593.

Strassburg CP, Strassburg A, Kneip S, Barut A, Tukey RH, Rodeck B, and Manns MP (2002) Developmental aspects of human hepatic drug glucuronidation in young children and adults. *Gut* **50:**259-265.

Wang H, Ren L, He Y, Wei Y, Chen Z, Yang W, Fu Y, Xu X, Fu W, Hu G, and Lou W (2014) Association between cytochrome P450 2C9 gene polymorphisms and colorectal cancer susceptibility: evidence from 16 case-control studies. *Tumour Biol* **35:**4317-4322.

Wauthier V, Verbeeck RK, and Buc Calderon P (2004) The use of precision-cut liver slices from male Wistar rats as a tool to study age related changes in CYP3A induction and in formation of paracetamol conjugates. *Toxicol In Vitro* **18:**879-885.

Wei P, Zhang J, Egan-Hafley M, Liang S, and Moore DD (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* **407:**920-923.

Whysner J, Ross PM, and Williams GM (1996) Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion. *Pharmacol Ther* **71:**153-191.

Xu C, Li CY, and Kong AN (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28:**249-268.

Footnotes

This work was supported by National Institute of Health (NIH) R01 Grants [ES019487 and GM111381], as well as start-up funds from University of Washington Center of Ecogenetics and Environmental Health (CEEH) [P30 ES0007033].

Figure Legends

Figure 1: The mRNA expression of Phase-I drug-metabolizing enzymes in livers of wild-type male mice after treatment with corn oil (vehicle), TCDD (AhR ligand), TCPOBOP (CAR ligand), or PCN (PXR ligand) at 2, 5, 25, and 60-days of age (n=4~6 per group). The expression measured in triplicate are first normalized to the expression of the housekeeping gene β-actin, and then are expressed as fold-changes compared to vehicle-treated group at that particular age. Asterisks (*) represent statistical difference ($p<0.05$) compared to the vehicle-treated group at the same age.

Figure 2: The mRNA expression of Phase-II Glutathione *S*-transferases (Gsts) enzymes in livers of wild-type male mice after treatment with corn oil (vehicle), TCDD (AhR ligand), TCPOBOP (CAR ligand), or PCN (PXR ligand) at 2, 5, 25, and 60-days of age (n=4~6 per group). The expression measured in triplicate are first normalized to the expression of the housekeeping gene β-actin, and then are expressed as fold-changes compared to vehicle-treated group at that particular age. Asterisks (*) represent statistical difference ($p<0.05$) compared to the vehicle-treated group at the same age.

Figure 3: The mRNA expression of more Phase-II enzymes in livers of wild-type male mice after treatment with corn oil (vehicle), TCDD (AhR ligand), TCPOBOP (CAR ligand), or PCN (PXR ligand) at 2, 5, 25, and 60-days of age (n=4~6 per group). The expression measured in triplicate are first normalized to the expression of the housekeeping gene β-actin, and then are expressed as fold-changes compared to vehicle-treated group at that

particular age. Asterisks (*) represent statistical difference ($p<0.05$) compared to the vehicle-treated group at the same age.

Figure 4: The mRNA expression of Phase-II UDP-glucuronosyltransferses (UGTs) enzymes in livers of wild-type male mice after treatment with corn oil (vehicle), TCDD (AhR ligand), TCPOBOP (CAR ligand), or PCN (PXR ligand) at 2, 5, 25, and 60-days of age (n=4~6 per group). The expression measured in triplicate are first normalized to the expression of the housekeeping gene β-actin, and then are expressed as fold-changes compared to vehicle-treated group at that particular age. Asterisks (*) represent statistical difference ($p<0.05$) compared to the vehicle-treated group at the same age.

Figure 5: The mRNA expression of Uptake (Oatp1a4) and Efflux (Mrp2, Mrp3, Mrp4) transporters in livers of wild-type male mice after treatment with corn oil (vehicle), TCDD (AhR ligand), TCPOBOP (CAR ligand), or PCN (PXR ligand) at 2, 5, 25, and 60-days of age (n=4~6 per group). The expression measured in triplicate are first normalized to the expression of the housekeeping gene β-actin, and then are expressed as fold-changes compared to vehicle-treated group at that particular age. Asterisks (*) represent statistical difference ($p<0.05$) compared to the vehicle-treated group at the same age.

Figure 6: The mRNA expression of xenobiotic-sensing transcription factors in livers of wild-type male mice after treatment with corn oil (vehicle), TCDD (AhR ligand), TCPOBOP (CAR ligand), or PCN (PXR ligand) at 2, 5, 25, and 60-days of age (n=4~6 per group). The expression measured in triplicate are first normalized to the expression of the

housekeeping gene β-actin, and then are expressed as fold-changes compared to vehicle-treated group at that particular age. Asterisks (*) represent statistical difference ($p<0.05$) compared to the vehicle-treated group at the same age.

Figure 7: Western blotting analysis of Mrp4, Oatp1a4, Cyp3a11 and Cyp2b10 proteins in livers of mice treated with corn oil, TCPOBOP, or PCN, at 5-, 25-, and 60-days of age (n= 3 per group). The intensities of protein bands were quantified using Image J software, and data were normal ized to the band intensity of β-actin protein. Asterisks (*) represent values statistically different from the corn oil-treated mice at the same age.

Figure 8: Enzyme activities of Cyp1a, Cyp2b and Cyp3a in liver microsomes treated with corn oil, TCPOBOP, or PCN, at 5-, 25-, and 60- days of age (n= 4~6 per group). The magnitude of the luminescent signal is measured in triplicate per group, and is proportional to the amount of luciferin product generated by the P450 reaction. Asterisks (*) represent values statistically different from the corn oil-treated mice at the same age.

DMD Fast Forward. Published on November 17, 2015 as DOI: 10.1124/dmd.115.066639
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Highest fold-induction of drug-processing genes among neonatal (Day 2 and Day 5), adolescent (Day 25) and adult (Day 60) ages

Ages	Ligand (Xenobiotic-sensors)	Drug-processing genes
Neonatal (Day 2 and 5)	TCDD (AhR)	Cyp1a2, Sult5a1, Ugt1a9
	TCPOBOP (CAR)	Cyp3a11, Mrp2
	PCN (PXR)	Gstm2, Gstm3
Adolescent (Day 25)	TCDD (AhR)	Ugt1a6, Mrp4
	TCPOBOP (CAR)	Cyp2b10, Ugt2b34, Ugt2b35
	PCN (PXR)	Gsta1, Gsta4, Sult1e1, Ugt1a1, Mrp3, Mrp4
Adult (Day 60)	TCPOBOP (CAR)	Aldh1a1, Aldh1a7, Ugt2b36
	PCN (PXR)	Papss2, Oatp1a4

Figure 1. Phase-I drug metabolizing enzymes RT-qPCR

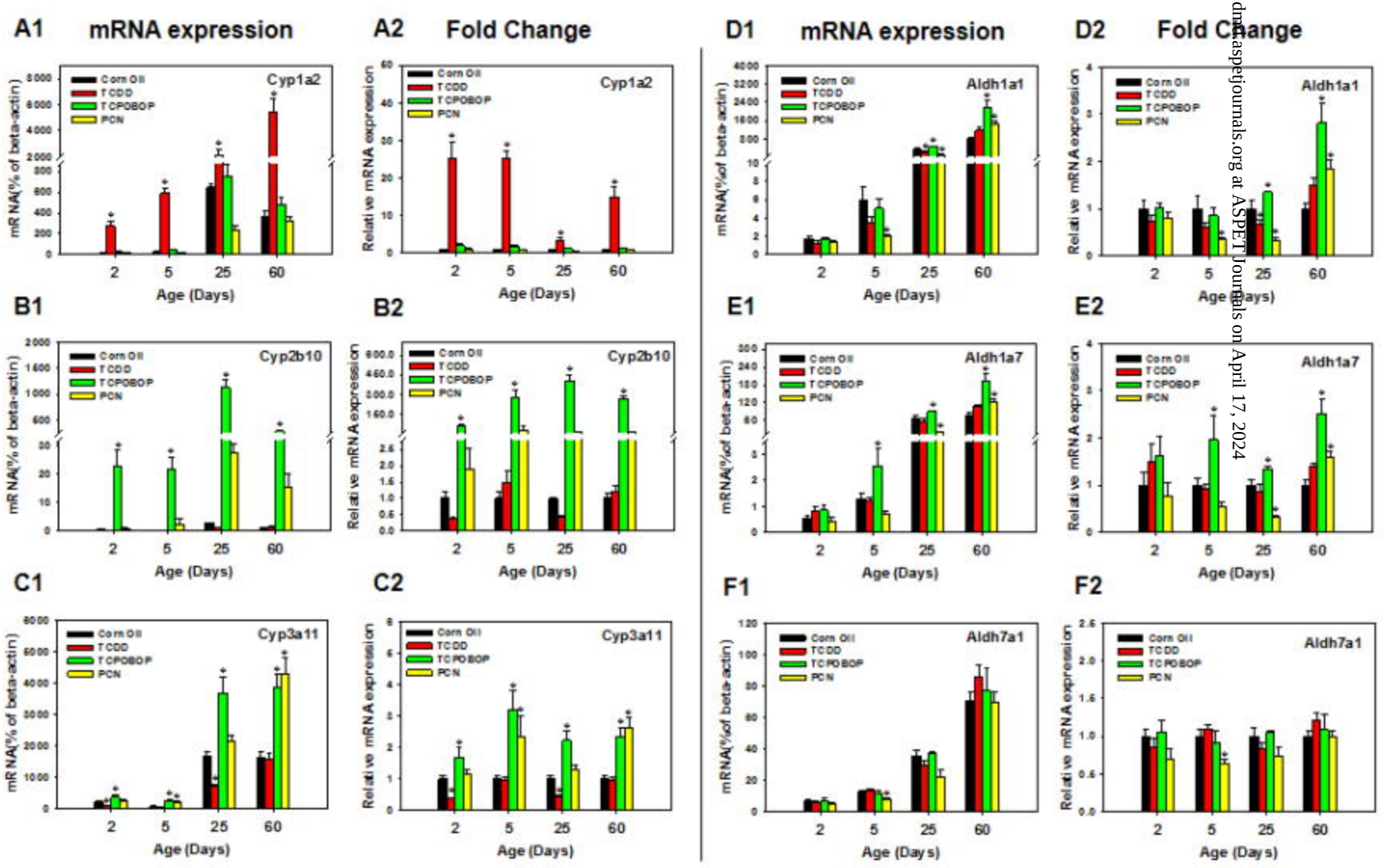


Figure 2. Phase-II drug metabolizing enzymes RT-qPCR

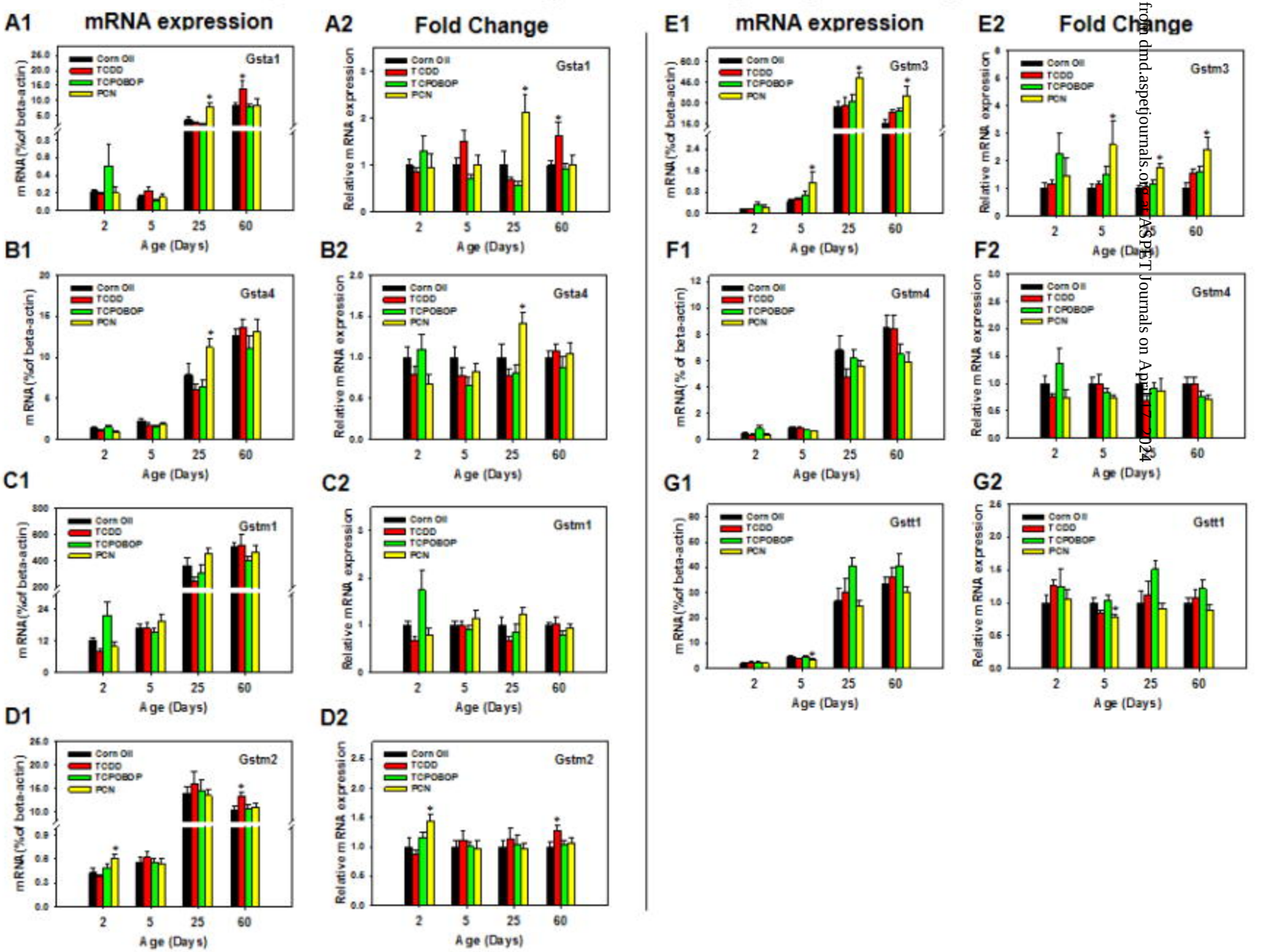


Figure 3. Phase-II drug metabolizing enzymes RT-qPCR

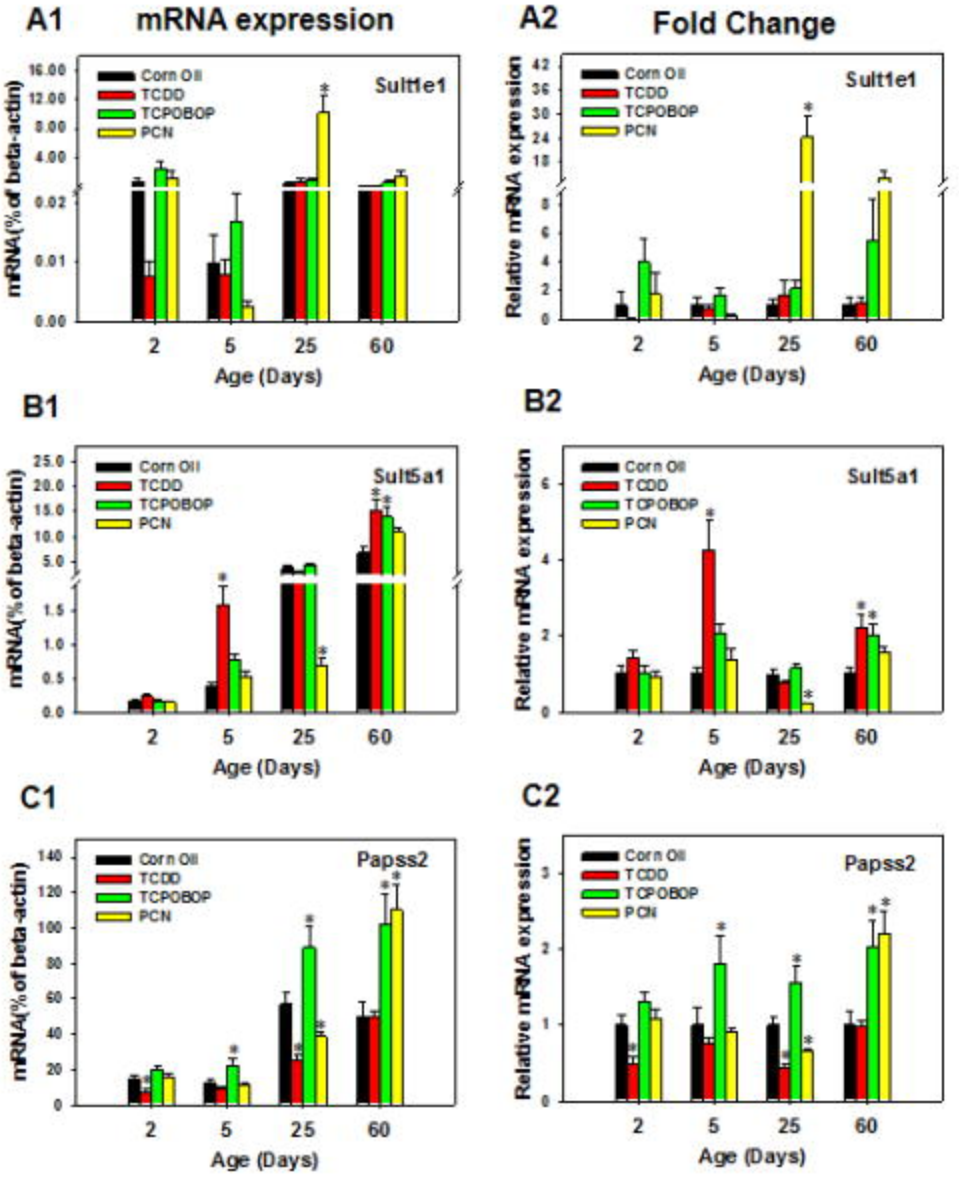


Figure 4. Phase-II drug metabolizing enzymes RT-qPCR

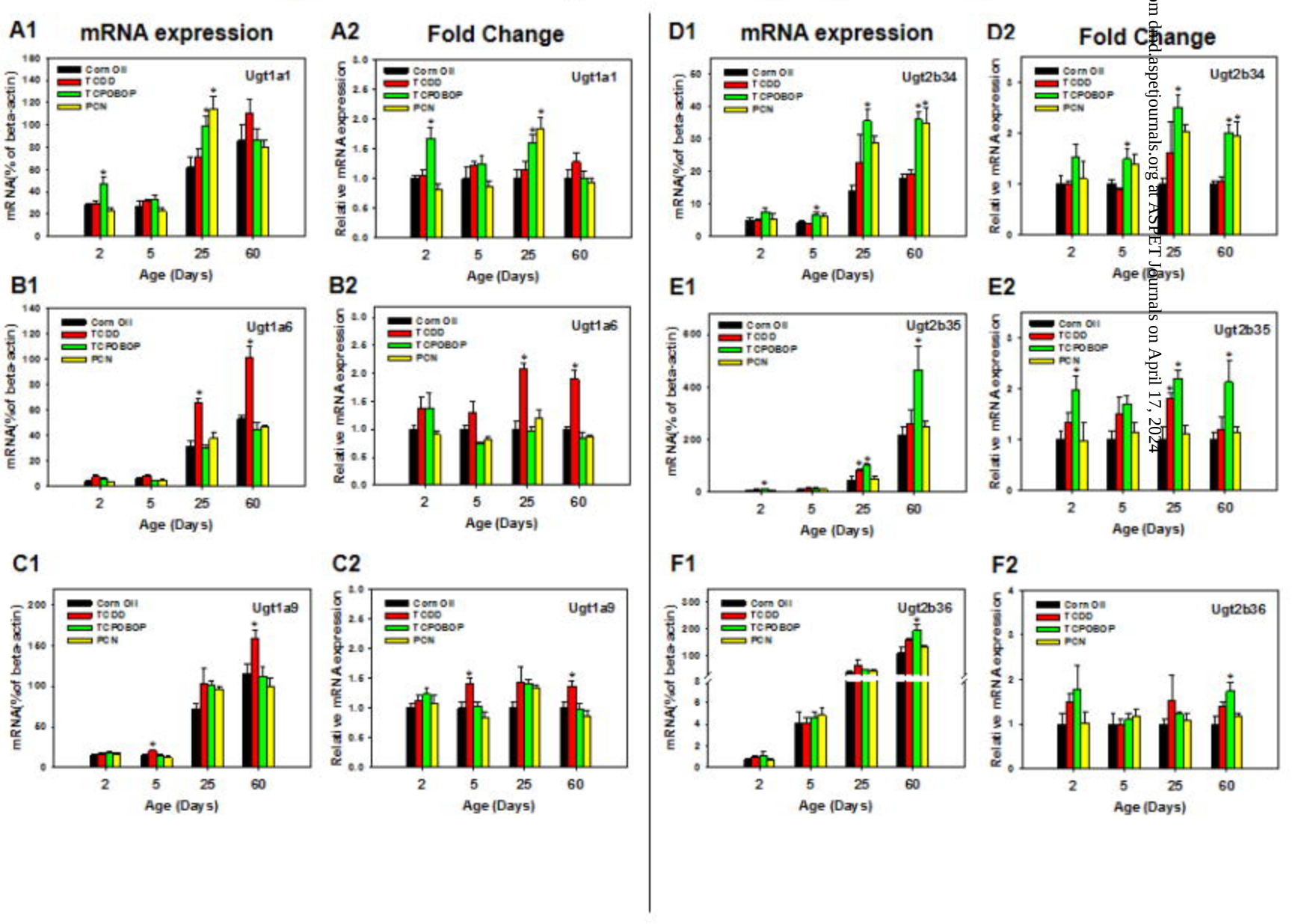


Figure 5. Transporters RT-qPCR

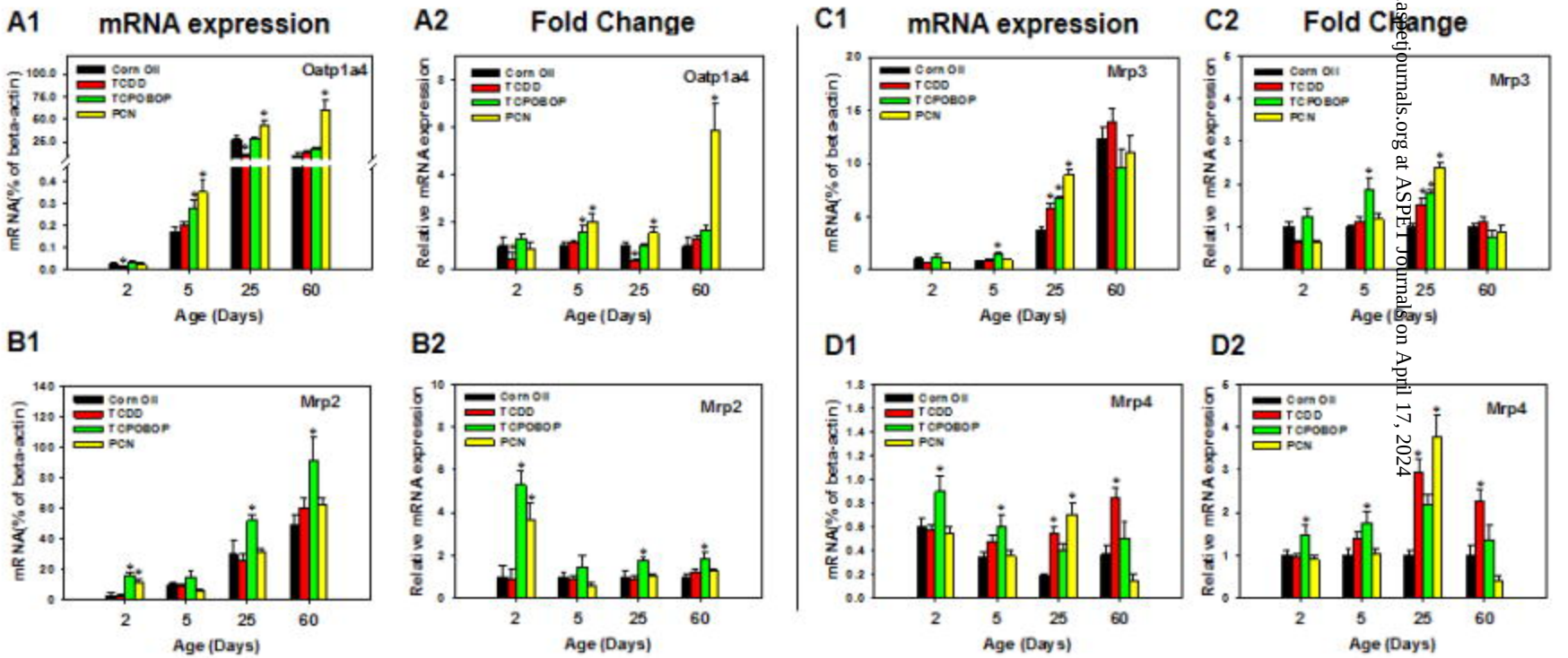


Figure 6. Xenobiotic sensors

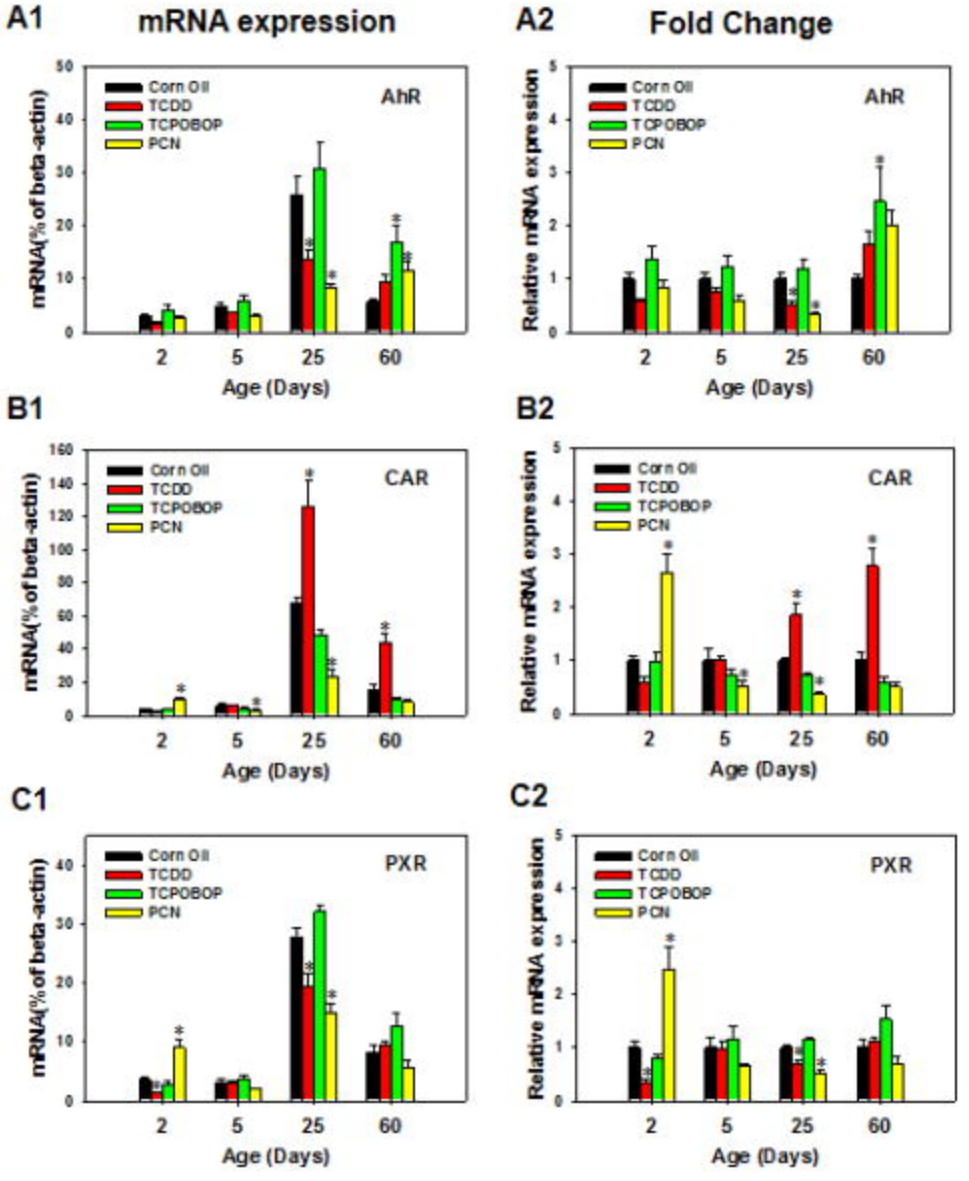
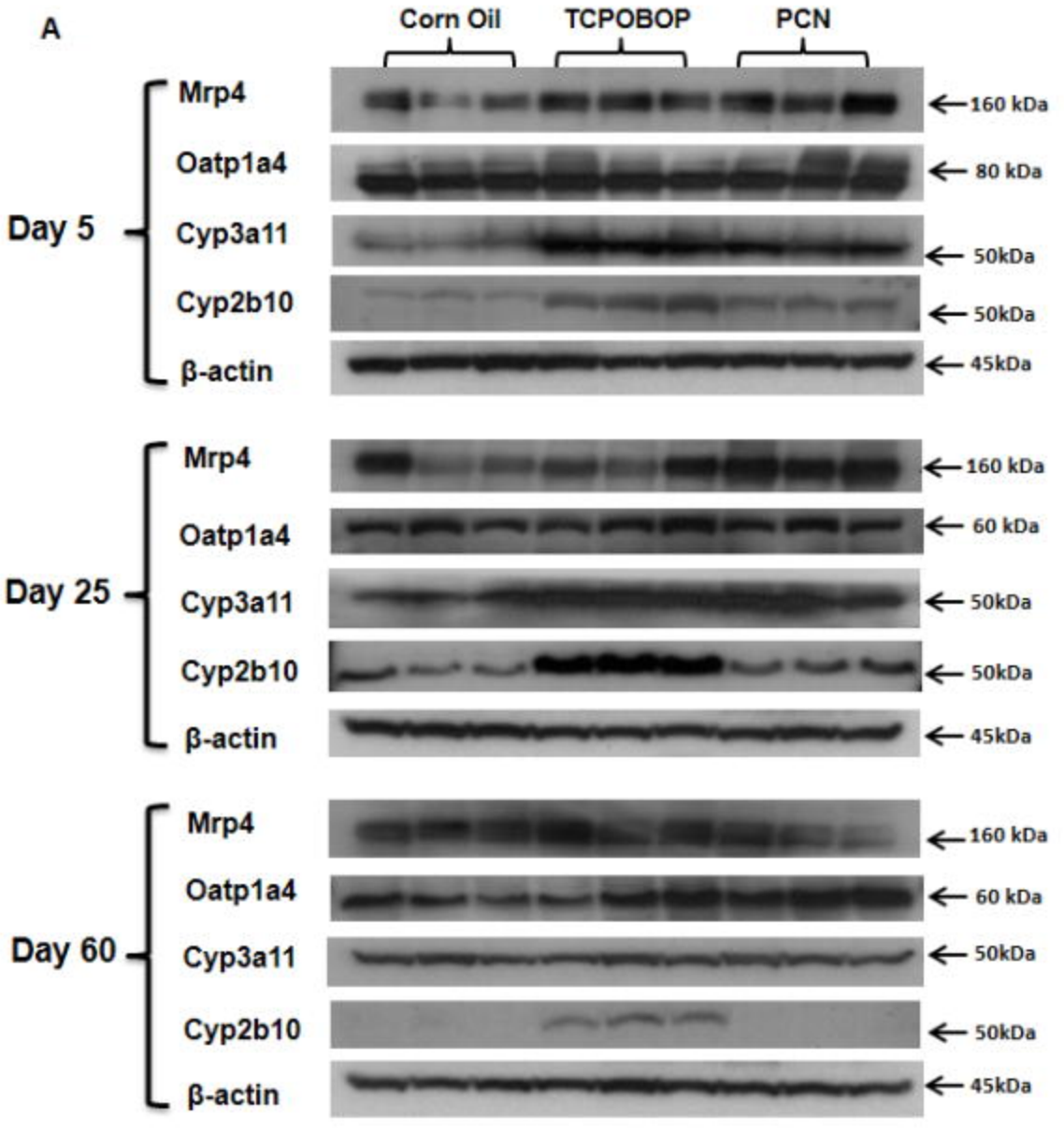
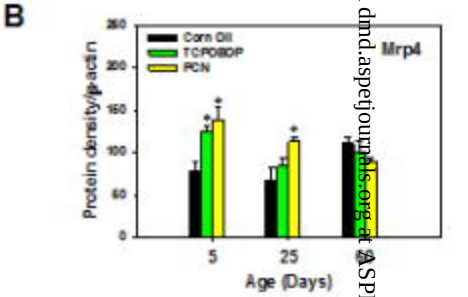
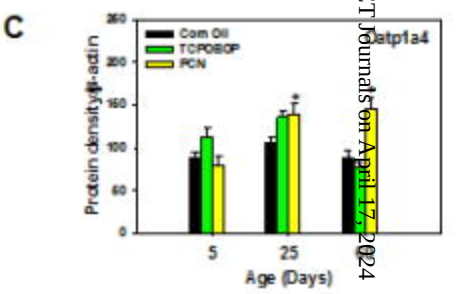
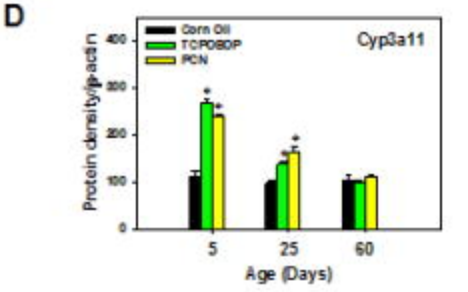


Figure 7. Protein expression









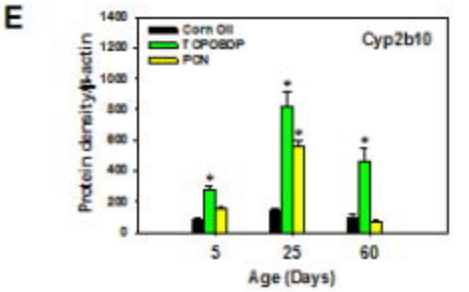


Figure 8. Enzyme activities