Triclosan Disrupts Thyroid Hormones: Mode-of-Action, Developmental Susceptibility, and Determination of Human Relevance

Katie Beth Paul

"A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Toxicology."

Chapel Hill
2011

Approved by:

Kim L. R. Brouwer, Pharm.D., Ph.D.
Kevin M. Crofton, Ph.D.
Michael J. DeVito, Ph.D.
Philip C. Smith, Ph.D
James A. Swenberg, D.V.M., Ph.D.

Abstract

Katie Beth Paul

Triclosan Disrupts Thyroid Hormones: Mode-of-Action, Developmental Susceptibility, and Determination of Human Relevance

(Under the direction of Kevin M. Crofton, Ph.D.)

Preliminary study demonstrated that triclosan (TCS), a bacteriostat in myriad consumer products, decreases serum thyroxine (T4) in rats. Adverse neurodevelopmental consequences result from thyroid hormone (TH) disruption; therefore determination of whether TCS disrupts THs during development, its mode-of-action (MOA), and the human relevance is critical. This research tested the hypothesis that TCS disrupts THs via activation of pregnane X and constitutive androstane receptors (PXR, CAR), mediating Phase I-II enzyme and hepatic transporter expression and protein changes, thereby increasing catabolism and elimination of THs, resulting in decreased TH concentrations. For Aim One, the hypothesized MOA was assessed using weanling female Long-Evans rats orally exposed to TCS (0-1000 mg/kg/day) for four days. Serum T4 decreased 35% at 300 mg/kg/day. Activity and expression of markers of Phase I (Cyp2b, Cyp3a1) and Phase II (Ugt1a1, Sult1c1) metabolism were moderately induced, consistent with PXR and/or CAR activation and increased hepatic catabolism. Susceptibility of dams and developing rats to TCS-induced hypothyroxinemia was determined for Aim Two. Long-Evans dams received TCS (0-300 mg/kg/day) orally from gestational day (GD) 6 to postnatal day (PND) 21; tissues were collected from fetuses (GD20), pups (PND4, 14, 21), and dams (GD20, PND22).

Serum T4 decreased 30% in GD20 dams and fetuses, PND4 pups, and PND22 dams (300 mg/kg/day). Minor increases in activity and expression of markers of hepatic Phase I (Cyp2b, Cyp3a) and Phase II (T4-glucuronidation in PND22 dams) metabolism were consistent with PXR/CAR activation and concomitant minor decreases in T4. For Aim Three, cell-based rat and human PXR and CAR reporter assays were employed to evaluate the human relevance of the putative MOA. TCS (10-30 µM) demonstrated human receptor reporter activities: inverse agonism of CAR1 and agonism of CAR2 CAR3, and PXR. TCS was an inverse agonist of rat CAR, similar to compounds that increase Phase I-II metabolism downstream. Although the data indicate potential species differences in the initiating key event, downstream effects on hepatic catabolism may be similar due to overlapping transcriptional regulatory functions of PXR and CAR. These data establish a plausible MOA for TCS-induced hypothyroxinemia in rats and demonstrate initiation of the MOA in human models.

To my uncle, Kenneth Marshall Paul (June 7, 1942 - August 21, 2010), a courier, New York City public transportation expert, lover of food and culture, and quietly hilarious friend to many. What he lacked in "book smarts" he made up for amply with his sense of humor and will (perhaps stubbornness to some). He was a testament to our shared belief that hard work and fulfilling contributions need not be limited by innate intelligence at birth. Despite his mental retardation, his efforts to survive, work, make friends, travel, attend Broadway shows, and give back, supported by the beliefs and help of my grandmother, Sophie Paul, made these things a reality. While by some unexplained event I was not born with the same disadvantage, this dissertation work is also the result of this common philosophy in my family: working hard, and even harder, makes it so.

Acknowledgements

First I would like to thank Kevin M. Crofton, PhD., for his invaluable mentorship throughout this work, and hopefully in the future. The time and patience he invested in me and this project will not be forgotten.

Considerable thanks are also due to my dissertation committee members: Kim L. R. Brouwer, Pharm.D., Ph.D., Michael J. DeVito, Ph.D., Philip C. Smith, Ph.D., and James A. Swenberg, D.V.M., Ph.D. for their help and guidance throughout the project. Dr. DeVito served as my laboratory co-mentor and spent extra time ensuring my success and asking helpful questions. Drs. Brouwer and Smith also hosted me in their laboratories for additional experimentation and training, which was exceedingly helpful not only to this work, but also my next career steps. Drs. Swenberg and Brouwer provided much additional support by their willingness to help me pursue additional outside funding resources.

Special thanks to Joan M. Hedge for her support and training during my time in the Crofton Lab at the US EPA. I benefitted greatly from her technical knowledge particularly of animal-handling, and also greatly enjoyed my time working with her on many projects.

Additional thanks to Steve Simmons, Ph.D., for providing the immense opportunity to work with him in his laboratory to develop Aim Three of this work; Geremy Knapp for his technical advice on PCR applications; David Ross, Elizabeth Boykin, and Joyce Royland, Ph.D., for training on equipment; previous lab member Joshua A. Harrill, Ph.D. for his support and advice; Tracy Marion, Ph.D.. Kristina Wolf, Ph.D., and David Harbourt, Ph.D. for training in UNC labs; Mary E. Gilbert, Ph.D., William R. Mundy, Ph.D., Douglas C. Wolf, D.V.M., Ph.D., Stephanie Padilla, Ph.D., and the many, many other people at the US

EPA who provided support and importantly access to many of the tools used in this work; the University of North Carolina at Chapel Hill Curriculum in Toxicology, particularly David J. Holbrook, Ph.D., Marila Cordeiro-Stone, Ph.D., and Julie Cannefax; Arlo Brown and Elaine Kimple of the UNC School of Pharmacy; the National Institute of Environmental Health Sciences Training Grant (T32-ES07126); the EPA/UNC Toxicology Research Program Training Agreement (CR833237); the US EPA/BASF Cooperative Research and Development Agreement (CRADA 546-09) that funded a portion of this work, as well as Robert Peter, Ph.D., Edgar Leibold, Ph.D., James Plautz, Ph.D., and Lisa Navarro, Ph.D. who enabled this agreement and provided useful discussions; the Society of Toxicology Colgate-Palmolive Award for Student Research Training in Alternative Methods; and the PhRMA Foundation Predoctoral Pharmacology/Toxicology Fellowship program.

Table of Contents

List of Tables……..…….....xii

List of Figures……..…….....xiii

List of Abbreviations and Gene Symbols……xv

Chapter 1: Introductory Chapter…..…..…….....1

1. Overview……...2

2. Triclosan applications in personal care and pharmaceutical products……..4

3. Environmental occurrence……5

4. Measured exposures to triclosan……...7

4a. Wildlife exposure……7

4b. Human exposure…….7

5. Absorption, distribution, metabolism, and excretion (ADME) of triclosan…….9

5a. Routes of exposure and absorption…….9

5b. Distribution, metabolism, and excretion……...10

6. Brief examination of toxicity in mammals…….11

6a. Dermal toxicity……..12

6b. Oral toxicity……..12

6c. Carcinogenicity…….13

6d. Reproductive and teratological toxicity……14

7. Evidence for triclosan-induced endocrine disruption and potential mechanisms……...14

7a. Evidence for triclosan-induced sex hormone disruption……...14

7b. Evidence for triclosan-induced thyroid hormone disruption……16

8. Thyroid hormones regulate physiology……..19

8a. Overview of regulation of the thyroid hormone system……...19

8b. Physiological action of thyroid hormones in adult organisms……..22

8c. Thyroid hormones during development……23

8d. Mechanisms of thyroid hormone disruption…….25

9. Rat as a model of thyroid hormone disruption……28

9a. Potential relevance to humans: differences in thyroid hormone homeostasis between rats and humans…….29

9b. Potential relevance to humans: evidence for thyroid disruption in humans by microsomal enzyme inducers……...31

10. Summary and human health significance…….33

11. Experimental Design…….33

12. References…….37

Chapter 2: Short-term Exposure to Triclosan Decreases Thyroxine *In Vivo* via Upregulation of Hepatic Catabolism in Young Long-Evans Rats**……..**……..……..55

1. Abstract……...56

2. Introduction…….57

3. Methods……...59

4. Results…….65

5. Discussion……...68

6. Supplementary Data……75

7. Funding Information……...75

8. Acknowledgments……...75

9. References……...86

Chapter 3: Developmental Triclosan Exposure Decreases Maternal and Neonatal Thyroxine in Rats ……...……..92

1. Abstract……...93
2. Introduction…….93
3. Methods……...95
4. Results…….98
5. Discussion…….100
6. Acknowledgements……...103
7. Funding…….103
8. References…….108

Chapter 4: Developmental Triclosan Exposure Decreases Maternal, Fetal, and Early Neonatal Thyroxine: A Toxicodynamic and Toxicokinetic Analysis..……...……...112

1. Abstract…….113
2. Introduction…….....114
3. Methods…….118
4. Results……...127
5. Discussion…….135
6. Conclusions……...145
7. Acknowledgements……...145
8. Funding…….146
9. References…….164

Chapter 5: Human Relevance of Triclosan-Induced Hypothyroxinemia: Evidence for Triclosan Activation of Human and Rodent Xenobiotic Nuclear Receptors……...……172

1. Abstract…….173
2. Introduction……...174

3. Methods……181

4. Results……...184

5. Discussion……189

6. Conclusions……...193

7. References……201

Chapter 6: Summary……211

Appendix 1: Short-Term *in Vivo* Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine……236

Appendix 2: Supplemental Data Table for Chapter 2……...250

Appendix 3: Supplemental Data Table for Chapter 3……...251

Appendix 4: Triiodothyronine (T3) Supplemental Data for Chapter 4……252

Appendix 5: Time-Course of Triclosan-Induced Cytotoxicity in DPX-2 and RPXR Receptor Reporter Cell Lines……..253

Appendix 6: Functional Hepatic Transport May Not Contribute Significantly to Triclosan-Induced Hypothyroxinemia in Rats: Description of Experiments and Results……..254

Appendix 7: Reconstituted Freeze-Dried Wastewater Effluents Activate the Human Pregnane-X Receptor in Receptor Reporter Assays in DPX-2 cells……279

List of Tables

Chapter 2:

Table 2.1 Summary of Taqman Gene Expression Assays and Amplification Efficiencies ……84

Table 2.2 Doses, group sizes, and block-controlled body weight gain, liver weight, and liver-to-body-weight ratio with standard deviation values ……85

Chapter 3:

Table 3.1 Gestation length, gender ratio, litter size, viability, and eye opening ……..106

Table 3.2 No-observed-effect-level and benchmark dose levels for T4 effects by age……..107

Chapter 4:

Table 4.1 Experimental blocks and n for each parameter measured ……..161

Table 4.2 Taqman Gene Expression Assays included in the Taqman Custom Low Density Array ……..162

Table 4.3. No-observed-effect level and benchmark dose levels for T4 decreases…….……..163

List of Figures

Chapter 1:

Figure 1.1 Triclosan is a chlorinated biphenyl ether similar to iodothyronines…..4

Chapter 2:

Figure 2.1 Triclosan decreases total serum thyroid hormones……..77

Figure 2.2 Dose-response curves for the effects of a 4-day exposure to triclosan on liver microsomal EROD and PROD activity……...78

Figure 2.3 Dose-response curves for the effects of a 4-day exposure to triclosan on liver RNA expression of CYP isoforms……..79

Figure 2.4 Dose-response curve for the effects of a 4-day exposure to triclosan on liver microsomal UGT activity……80

Figure 2.5 Dose-response curve for the effects of a 4-day exposure to triclosan on liver mRNA expression of UGT isoforms ……81

Figure 2.6 Dose-response curve for the effects of a 4-day exposure to triclosan on liver mRNA expression of SULT isoforms……82

Figure 2.7 Dose-response curve for the effects of a 4-day exposure to triclosan on liver mRNA expression of transporters……..83

Chapter 3:

Figure 3.1 Dam body weight during the gestational and lactation periods……..…104

Figure 3.2 Percent of control serum total T4 for dams and pups……105

Chapter 4:

Figure 4.1 Dosing and tissue collection schedule from GD6 through PND22…….147

Figure 4.2 Percent of control T4for dams and offspring……148

Figure 4.3 Percent of control serum TSH for dams and offspring……..…149

Figure 4.4 Percent of control hepatic microsomal EROD and PROD activity for dams and offspring compared to controls……..150

Figure 4.5 Hepatic microsomal UGT-T4 activity……...151

Figure 4.6 Hepatic CYP gene expression……...152

Figure 4.7 Hepatic Phase II gene expression……..154

Figure 4.8 Hepatic TH-responsive gene expression……155

Figure 4.9 Positive control Phase I, Phase II, Phase III, and TH-responsive gene expression……..…156

Figure 4.10 Heatmap of hepatic gene expression by time point and TCS exposure group (mg/kg/day)……..157

Figure 4.11 Mean total, parent, and percent parent TCS content in sera and liver…….158

Figure 4.12 TCS content in sera and liver versus serum T4 concentrations at 300 mg/kg/day……...…160

Chapter 5:

Figure 5.1 hPXR activation in DPX2 cells at 6, 12, 24, and 48 hr of chemical exposure…….195

Figure 5.2 rPXR activation in RPXR cells at 6, 12, 24, and 48 hr of chemical exposure…….196

Figure 5.3 Comparative activation of full-length PXR for human and rat……..…197

Figure 5.4 Comparative CYP3A4 activation for human and rat in the full-length receptor reporter cell lines……...198

Figure 5.5 Activation of chimeric human and mouse PXR …….199

Figure 5.6 Activation of the chimeric hCAR by splice variant ……200

Figure 5.7 Activation of chimeric rat and mouse CAR ……..….201

List of Abbreviations and Gene Symbols

Actb:	Actin, beta
AhR:	Aryl hydrocarbon receptor
BMD:	Benchmark dose
BMR:	Benchmark response
CITCO:	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime
CTZ:	Clotrimazole
CYP:	Cytochrome P450
Cyp1a1:	Cytochrome P450, family 1, subfamily a, polypeptide 1
Cyp2b1/2:	Cytochrome P450, family 2, subfamily b, polypeptide 2
Cyp3a1/23:	Cytochrome P450, subfamily 3A, polypeptide 1
Cyp3a2:	Cytochrome P450, subfamily 3A, polypeptide 2
Cyp3a9:	Cytochrome P450, subfamily 3A, polypeptide 9
Cyp4a2:	Cytochrome P450, subfamily 4A, polypeptide 2
DEHP:	di-(2-ethylhexyl)phthalate
DioI:	Deiodinase, iodothyronine, type I
EC50:	Half-maximal effect concentration
EROD:	Ethoxyresorufin-O-deethylase
Gapdh:	Glyceraldehyde-3-phosphate dehydrogenase
GD:	Gestational day
hCAR:	Human constitutive androstane receptor
hPXR:	Human PXR
LOAEL:	Lowest-observed-adverse-effect level

LOEL: Lowest-observed-effect level

mCAR: Mouse constitutive androstane receptor

Mct8: solute carrier family 16 (monocarboxylic acid transporters), member 2

Mdr1b: ATP-binding cassette, subfamily B (MDR/TAP), member 1B

MeI: Malic enzyme 1, NADP(+)-dependent, cytosolic

MOA: Mode-of-action

mPXR: Mouse PXR

mRNA: messenger ribonucleic acid

Mrp2: ATP-binding cassette, subfamily C (CFTR/MRP), member 2

NOAEL: No-observed-adverse-effect level

NOEL: No-observed-effect level

Oatp1a1: Solute carrier organic anion transporter family, member 1a1

Oatp1a4: Solute carrier organic anion transporter family, member 1a4

PB: Phenobarbital

PBDEs: Polybrominated diphenyl ethers

PCBs: Polychlorinated biphenyl ethers

PND: Postnatal day

PPARα: Peroxisome proliferator activated receptor alpha

PROD: pentoxyresorufin-O-deethylase

qPCR: Quantitative Polymerase Chain Reaction

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

rCAR: Rat constitutive androstane receptor

RIA: Radioimmunoassay

Rpl13a:	Ribosomal protein L13A
Rps18:	Ribosomal protein S18
rPXR:	Rat PXR
SULT:	Sulfotransferase
Sult1b1:	Sulfotransferase family 1B, member 1
Sult1c1:	Sulfotransferase family 1C, member 1
Sult1c3:	Sulfotransferase family 1C, member 3
T3:	Triiodothyronine
T4:	Thyroxine
TCDD:	2,3,7,8-tetrachlorodibenzodioxin
TCPOBOP:	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TCS:	Triclosan
TH:	Thyroid hormone
Thrsp:	Thyroid hormone-responsive
UGT:	Glucuronyltransferase
Ugt1a1:	UDP-glycosyltransferase 1 family, polypeptide A1
Ugt1a6:	UDP-glycosyltransferase 1 family, polypeptide A6
Ugt2b:	UDP-glucuronosyltransferase 2 family
Ugt2b5:	UDP-glucuronosyltransferase 2 family, member 5

Characterization of the mode-of-action, developmental susceptibility, and human relevance of triclosan-induced hypothyroxinemia in the rat

Introductory Chapter 1

Katie B. Paul[1]

[1]University of North Carolina at Chapel Hill, Curriculum in Toxicology, CB 7270, Chapel Hill, NC 27599

1. Overview

Triclosan (2,4,4'-trichloro-2'-hydroxyphenylether) (TCS) is a chlorinated phenolic bacteriostat found as an active ingredient in many personal care products, including items such as shower gels, soaps,deodorants, and toothpastes(Bhargava *et al.*, 1996; DeSalva *et al.*, 1989), and in a majority of liquid hand soaps sold in the US (Perencevich *et al.*, 2001). Availability of environmental TCS concentrations and ecotoxicity data initiated important questions about the potential non-target toxicity of TCS. Measurements confirming parts per trillion (ng/L) to low parts per billion (µg/L) TCS concentrations in surface waters and wastewater treatment plant effluents in the United States and other countries (Kanda *et al.*, 2003; Kolpin *et al.*, 2002; Lopez-Avila, 1980; Paxeus, 1996; Singer *et al.*, 2002) coincided with reports of systemic toxicity to microorganisms, daphnids, fish, and algae (Orvos *et al.*, 2002; Singer et al., 2002). Aquatic reproductive toxicity studies suggested TCS acted as a weak androgen (Foran *et al.*, 2000) or estrogen in medaka (*Oryzias latipes*) (Ishibashi *et al.*, 2004), or anti-estrogen in male South African clawed frogs (*Xenopus laevis*) (Ishibashi et al., 2004; Matsumura *et al.*, 2005; Tatarazako *et al.*, 2004). Particularly interesting was a report that TCS exposure resulted in advanced thyroid-hormone dependent metamorphosis and altered gene expression of thyroid receptor β (TRβ) in triiodothyronine (T3)-stimulated frogs (Veldhoen *et al.*, 2006). These endocrine-related effects suggested an impact on thyroid hormone homoestasis in frogs, but the effects of TCS on thyroid hormone homeostasis in mammals remained to be elucidated.

Initial study of TCS exposure using a 4-day short-term assay paradigm, used previously to identify thyroid hormone disruptors such as polyaromatic hydrocarbons and

polychlorinated biphenyls (Craft *et al.*, 2002; Crofton *et al.*, 2005a; Zhou *et al.*, 2001) produced dose-dependent decreases in thyroxine (T4) in weanling female Long Evans rats (Crofton *et al.*, 2007). These authors suggested that TCS might increase hepatic catabolism and elimination of T4, resulting in the observed serum T4 concentration decreases (Crofton et al., 2007). Disruption of thyroid hormones during development adversely affects neurodevelopment in both rats (Berbel *et al.*, 2010; Lavado-Autric *et al.*, 2003; Zoeller *et al.*, 2000; Zoeller *et al.*, 2007a) and humans (Haddow *et al.*, 1999; Kooistra *et al.*, 2006; Pop *et al.*, 2003; Pop *et al.*, 1999). Therefore it is critical to determine whether TCS disrupts thyroid hormones during development, to characterize its mode-of-action, and to discern the human relevance of this mode-of-action using comparative *in vitro* models. **This research tests the global hypothesis that TCS induces thyroid hormone disruption via activation of hepatic nuclear receptors, mediating downstream Phase I- Phase III xenobiotic metabolizing enzyme expression and protein changes, resulting in decreased circulating thyroid hormone concentrations in rats.**

This research was comprised of three Specific Aims. For Specific Aim 1, the effects of TCS on hepatic catabolism and transport in rats following a short-term exposure were determined *in vivo* using hepatic microsomal activity and mRNA expression, and *in vitro* using functional activity assays in sandwich-cultured rat hepatocytes. Sensitivity of the developing organism to TCS-induced thyroid hormone disruption via maternal TCS exposure, and the plausibility of the hypothesized MOA from Specific Aim 1, was evaluated in Specific Aim 2. The potential human relevance of the hypothesized MOA was determined in Specific Aim 3 by assessing activation of the constitutive androstane receptor and pregnane X receptor by TCS in both rat and human receptor-reporter assays.

These data provide the first report of TCS-induced thyroid hormone changes and the doses of TCS necessary to elicit effects across multiple developmental life-stages. Importantly, this work evaluates a potential MOA in rats that can be used to identify shared key events across species. Further, this work contributes to a growing understanding of species-specific nuclear receptor-regulated hepatic metabolic mechanisms.

2. Triclosan applications in personal care and pharmaceutical products

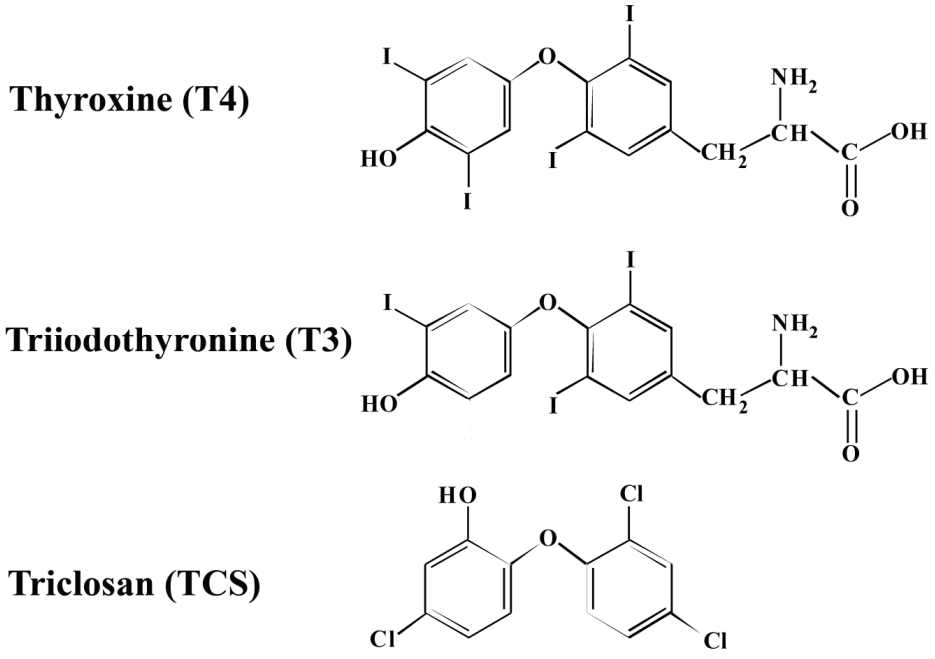


Figure 1.1. TCS is a chlorinated biphenyl ether structurally similar to iodothyronines.

TCS is a chlorinated biphenyl ether (Figure 1) with antimicrobial and antifungal properties that have made it a useful additive for myriad personal care and medical products for approximately forty years (Bhargava et al., 1996; Fang *et al.*, 2010; Waltman *et al.*, 2006). The biphasic action of TCS as a bacteriostat and bacteriocide depends upon the application concentration, with higher concentrations of TCS eliciting lethal effects on bacteria and fungi via disruption of cellular membranes (Guillen *et al.*, 2004; Yazdankhah *et al.*, 2006). The bacteriostatic activity of TCS results from inhibition of the FabI-associated

enoyl-acyl carrier protein reductase, preventing bacterial fatty acid elongation in susceptible bacteria and fungi species (Heath *et al.*, 2000; Levy *et al.*, 1999; McMurry *et al.*, 1998a). The resultant efficacy of adding TCS to soaps in household and hospital settings is a matter of debate (Aiello *et al.*, 2007; Gordin *et al.*, 2005; Haas *et al.*, 2005; Sickbert-Bennett *et al.*, 2005), in particular with respect to the risk of promoting antibiotic-resistance via modulation of several modes of antibiotic resistance (McMurry *et al.*, 1998b; Saleh *et al.*, 2011; Yazdankhah et al., 2006).

TCS is present in the majority of liquid hand soaps sold in the U.S. (Perencevich et al., 2001). The U.S. Department of Health and Human Services Household Product Database lists common personal care products containing TCS at 0.0015 to 1% of the product formulation, including deodorants, hand soaps, pet shampoos, dish soap, Colgate Total ® toothpaste, and liquid lipstick (http://householdproducts.nlm.nih.gov). In addition to personal care products, TCS is incorporated into dentrifices and other dental applications, impregnated into plastic products, clothing, and toys, and included in many surfaces under the commercial name, Microban® (Bhargava et al., 1996; Rodricks *et al.*, 2010).

3. Environmental occurrence

The use of TCS as a broad spectrum antibacterial agent (Heath et al., 2000) in many commercial and household products has resulted in widespread contamination of anthropogenic and natural environments (Kolpin et al., 2002). By some estimates, 96% of the consumer products containing TCS are washed down residential drains (Reiss *et al.*, 2002). Although wastewater treatment plant (WWTP) processes remove 70-98% of the TCS load (Bock *et al.*, 2010), this incomplete removal constitutes the primary route for TCS entrance into surface waters and the environment. TCS surface water concentrations in the

ng/L range (parts per trillion) have been confirmed across the U.S. (Chalew *et al.*, 2009; Loraine *et al.*, 2006; McAvoy *et al.*, 2002; Waltman et al., 2006) and abroad (Nakada *et al.*, 2006; Nishi *et al.*, 2008; Singer et al., 2002). In a nationwide study of impacted surface water sites from 1999-2000, TCS was one of the most often detected pollutants measured; TCS was detected at nearly 60% of tested sites, with an average concentration estimated at 50 ng/L for TCS when it was detected (Kolpin et al., 2002). The high octanol to water coefficient of both the ionized and protonated forms of TCS (pK_a estimates = 7.9 - 8.14) suggests high sediment sorption and low mobility (Orvos et al., 2002; Singer et al., 2002). Land-applied biosolids may also redistribute a minute amount of TCS back to the aquatic environment, due to high average concentrations of 4280 µg/kg sediment in biosolids derived from WWTP processes (Langdon *et al.*, 2010; Singer et al., 2002). Another small contributor to environmental occurrence may be bioaccumulation; limited bioaccumulation is indicated by the relatively low bioaccumulation factors estimated for algae (900-2700) (Coogan *et al.*, 2007) and fish (2500-4157) (Orvos et al., 2002) when compared to known bioaccumulative toxicants like Aroclor and chlordane, which have bioaccumulation factors in fish that are 1 to 2 orders of magnitude higher (30,000 – 274,000) (Orvos et al., 2002). The TCS metabolite methyl-TCS may have more bioaccumulative potential; bioaccumulation factors for methyl-TCS were estimated to be 100,000-260,000, suggesting a higher bioaccumulative potential (Balmer *et al.*, 2004). Clearly the environmental occurrence of TCS at parts per trillion concentrations stems from anthropogenic use of consumer products, but characterization of the distribution in the aquatic, and in particular terrestrial, environment is the subject of ongoing research.

4. Measured exposures to TCS

4a. Wildlife exposure

Several studies of aquatic species have demonstrated wildlife exposure to TCS in surface waters downstream of WWTPs or otherwise impacted waterways, demonstrating that TCS is present in the environment and environmental exposures may contribute to overall human exposure. TCS exposure of fish has been confirmed by measurements of TCS in fish bile, ranging from 0.24 to 25 ng/g, depending on species and distance from a WWTP (Adolfsson-Erici *et al.* 2002). An analysis of lean fish fillet tissue collected in Texas demonstrated a similar range of TCS concentrations, 17-31 ng/g (Mottaleb *et al.*, 2009); TCS was detected in fish fillet and liver collected from five other U.S. sites, but at concentrations below the detection limit of 38 ng/g (Ramirez *et al.*, 2009). In Swedish lakes, the metabolite methyl-TCS was detected at 35 ng/g in fish (Balmer *et al.*, 2004).

4b. Human exposure

Measurements of TCS in human serum (Allmyr *et al.*, 2008; Allmyr *et al.*, 2006b; Allmyr *et al.*, 2009; Hovander *et al.*, 2002), breast milk (Adolfsson-Erici *et al.*, 2002; Allmyr *et al.*, 2006a; Dayan, 2007), and TCS metabolites in urine (Calafat et al., 2008; Wolff et al., 2007) demonstrate that humans are exposed. Concentrations of TCS in human serum have ranged from 0.4 to 296 µg/kg wet weight in studies of the Swedish and Australian populations (Allmyr et al., 2006a; Allmyr et al., 2008; Allmyr et al., 2009). A survey of Australian volunteers demonstrated slightly higher average serum values (4.1-19 µg/kg) than in Sweden where there are general advisories against TCS use (Allmyr et al., 2008). The results from this study of the Australian population suggest relatively homogeneous exposure throughout territory, age group, and gender, with only slightly higher serum TCS concentrations

correlated with males aged 31-45 (Allmyr et al., 2008). A study of Swedish nursing mothers revealed that TCS concentrations in the serum of these mothers was 0.010 to 38 µg/kg fresh weight (Allmyr et al., 2006a). Of particular interest is that the TCS partitioned into serum at a much greater rate than into breast milk of these volunteers (Allmyr et al., 2006a). For these mothers, TCS was detected in the women who had been exposed to personal care products containing TCS at much higher concentrations than mothers who had not been exposed to these types of products (Allmyr et al., 2006a). However, since TCS was present in all mothers regardless of intentional personal care product exposure, this indicates that potentially unaccounted sources of exposure contribute to the total TCS exposure of the human population. The range of TCS detected in the breast milk of the mothers in this study, both control and exposed, was 0.018 µg/kg-lipid to 0.95 µg/kg-lipid (Allmyr et al., 2006a). In another study of five breast milk samples from Sweden, TCS concentrations ranged from less than 20 µg/kg-lipid to 300 µg/kg-lipid (Adolfsson-Erici et al., 2002). A study of 62 breast milk samples from milk donation banks in California and Texas demonstrated a range of TCS concentrations from below the limit of detection to 2100 µg/kg-lipid, with the majority of samples containing 200 µg/kg-lipid or less (Dayan, 2007). Based on these findings, and the standard volume of milk consumed by infants (Butte *et al.*, 1984; Butte *et al.*, 2002; EPA, 1997), Dayan (2007) used a conservative, high estimate of breast milk TCS concentration (1742 µg/kg-lipid) to estimate the maximum infant exposure to TCS as approximately 7.4 µg/kg/d. These findings underscore the potential for human developmental exposure via breast milk, when the potential impact of thyroid hormone disruption may be most deleterious (Miller *et al.*, 2009).

Urinary markers of TCS exposure further demonstrate widespread TCS exposure. A 2004-2005 study of girls from three U.S. metropolitan areas found mean urinary TCS concentrations of 17.1 µg/g-creatinine (10.0 µg/L when not corrected for creatinine) (Wolff et al., 2007). A broader National Health and Nutrition Examination Survey (NHANES) study of the U.S. population detected TCS above the LOD in approximately 75% of samples, and reported the mean and 95th percentile as 13.0 µg/L (12.7 µg/g-creatinine) and 459 µg/L (363.8 µg/g-creatinine), respectively (Calafat et al., 2008). Significant correlations between urinary TCS concentrations and age and socioeconomic status suggested that concentrations peaked for individuals in their thirties of greater economic means (Calafat et al., 2008). A comparison of the magnitude of these spot test urinary TCS concentrations with the known rate of cumulative urinary excretion, 44-57% at 48 hr after exposure (DeSalva *et al.*, 1989; Sandborgh-Englund *et al.*, 2006), would suggest that the order of magnitude for daily human exposure is in the ng /kg/day range. This is consistent with U.S. EPA conservative estimates of potential oral exposure from water sources (USEPA, 2008). TCS was detected in drinking water sources in Southern California at 49 ng/L (Loraine et al., 2006); assuming that this is the concentration of tap water for human consumption, this would be roughly equal to 98 ng/day (2L of water per day), or 1.4 ng/kg/day (average person of 70 kg) (USEPA, 2008).

5. Absorption, distribution, metabolism, and excretion (ADME) of TCS

5a. Routes of exposure and absorption

TCS is readily absorbed from the gastrointestinal tract and oral mucosa (Bagley *et al.*, 2000; Lin, 2000), and oral exposure is considered to be the predominant route of exposure (Sandborgh-Englund *et al.*, 2006). Dermal exposure may supplement oral exposure to TCS. The chlorinated phenolic structure (Figure 1) and high octanol/water partition coefficient at

neutral pH (log K_{ow} = 4.76), and a pK_a = 7.9 (Allmyr *et al.*, 2006b) suggests a potential for dermal absorption. Topical application results in TCS binding to the stratum corneum and continued bacteriostatic action against native and transient skin bacteria (Bhargava et al., 1996). Evidence for dermal absorption in the rat over 24 hours was 23% of the applied dose, while human absorption was 6.3%, suggesting that like other chemicals, dermal absorption in rats is higher than in humans (Moss *et al.*, 2000). The leading source of oral human exposure is likely the use of personal care products (Allmyr et al., 2006a), but surface water and food source contamination may contribute to this exposure .

5b. Distribution, metabolism, and excretion

Limited available kinetic studies support rapid elimination of TCS from mice and rats, and enterohepatic circulation and biliary elimination of TCS (Rodricks et al., 2010). There may be a species-specific distribution profile for TCS, as mice appear to accumulate TCS in the liver whereas rats do not (Rodricks et al., 2010). Alhough TCS tissue distribution into multiple compartments has not been reported in humans, serum concentrations of TCS following extended human exposures further supports a lack of accumulation of TCS following exposure (Bagley et al., 2000; Rodricks et al., 2010).

Glucuronidation and sulfation comprise the two major modes of mammalian TCS metabolism. The predominant pathway for metabolism of TCS in guinea pigs and rats is glucuronidation (Black *et al.*, 1975; Calafat et al., 2008; Moss et al., 2000), although TCS may also be hydroxylated at any of the chlorinated sites on the molecule (Tulp *et al.*, 1979). Metabolism of TCS to the glucuronide and sulfate conjugates occurs locally in skin, with TCS-glucuronide diffusing well into the skin of rats and humans within several hours (Black *et al.*, 1975). Human TCS metabolism proceeds through glucuronidation and sulfation

(Calafat et al., 2008; Sandborgh-Englund et al., 2006) , though sulfation may be the primary pathway (DeSalva et al., 1989). Conjugation of TCS also occurs in skin following dermal application (Black et al., 1975; Moss et al., 2000).

The human half-life of TCS is 21 hours as determined by a study comprised of ten volunteers who received one oral 4 mg dose, with a rapid absorption phase of 1-3 hours, and primarily urinary excretion of TCS and glucuronide and sulfate metabolites (Sandborgh-Englund et al., 2006). In contrast to previous studies where serum TCS appeared to be completely conjugated (DeSalva et al., 1989), Sandborgh-Englund *et al.* found that 30-35% of the total TCS was unconjugated in serum (DeSalva et al., 1989; Sandborgh-Englund et al., 2006); this difference may have been due to differences in exposure duration and sampling time or dose used. The half-life of TCS in rats is fairly comparable at 10-18 hrs, depending on exposure route and study cited (Black et al., 1975; Rodricks et al., 2010; Siddiqui *et al.*, 1979).

6. Brief examination of toxicity in mammals

Overall TCS demonstrates low toxicity by traditional metrics of adverse effects. TCS has a high acute LD50 dose in mammals, and does not produce any measurable overt toxicity in the population following routine use, and has a low potential for inducing dermal irritation. An earlier review of industry studies of human exposure to TCS through dental hygiene products demonstrated no adverse effects following routine and prolonged exposures to products containing 0.01 to 0.6% TCS (DeSalva et al., 1989). Further review of safety studies of TCS-containing dental hygiene products suggest that TCS has no systemic toxicity when exposures increase up to 30 mg/kg/day or when TCS-containing dentrifices are used for periods of years (Rodricks et al., 2010).

6a. Dermal toxicity

Numerous studies performed by Ciba-Geigy, Inc. of single-dermal application exposures to TCS preparations in rats and humans did not reveal any acute toxicity or sensitization when TCS comprised less than or equal to 1.5% of the formulation (Barbolt, 2002; Bhargava et al., 1996; Lyman *et al.*, 1969). Typically consumer products contain 0.01 to 0.3% TCS (Bhargava et al., 1996).

However, repeated topical application of preparations with greater than 1.5% in rats and mice resulted in dermal irritation (Lyman et al., 1969; Rodricks et al., 2010) and in rabbits with preparations containing 3% TCS (Moss et al., 2000). Only limited sporadic instances of dermal irritation have been reported following human use (Bhargava et al., 1996).

6b. Oral toxicity

TCS demonstrates extremely low acute oral toxicity in rats, rabbits, dogs, or humans, with demonstrated safety for use in dental products in humans (DeSalva *et al.*, 1989). The oral LD_{50} in rats for TCS is 3750-5000 mg/kg, and by subcutaneous injection route the LD_{50} climbs to greater than 14,700 mg/kg; chemicals with LD_{50} values greater than 2000 mg/kg are considered acutely nontoxic (Barbolt, 2002; Lyman *et al.*, 1969). The no observable effect level for clinical toxicity in a subchronic 90-day oral exposure in rats was 50 mg/kg/day, and 30 mg/kg/d for baboons (Barbolt, 2002; DeSalva et al., 1989).

Increasing exposure to subacute, subchronic, and chronic durations in murine models suggests that TCS increased liver weight and produced adaptive hepatic responses such as centrilobular hypertrophy, consistent with induction of Phase I cytochrome P450 enzymes and Phase II metabolic enzyme, with no other substantial effects noted in reviews of many

studies (Bhargava et al., 1996; Rodricks et al., 2010), with the exception of the production of liver tumors in mice only (Rodricks et al., 2010).

6c. Carcinogenicity

A preponderance of data confirm that TCS is nonmutagenic and nongenotoxic. Negative results in the Ames test, *in vitro* mammalian point mutation assay, *in vivo* chromosomal aberration assay, and unscheduled DNA synthesis assay in rat hepatocytes (Barbolt, 2002; Rodricks et al., 2010) provide consistent evidence that TCS does not cause genotoxic effects. However, the results of carcinogenicity studies in animals are stratified by species, with mice demonstrating differential susceptibility to induction of liver tumors via activation of the peroxisome proliferator-activated receptor alpha (PPARα), a tumor pathway not thought to be relevant for the development of human liver tumors (Gonzalez *et al.*, 2008; Klaunig *et al.*, 2003; Rodricks et al., 2010). In an eighteen-month carcinogenicity assay, an increased incidence of hepatocellular adenoma and/or carcinoma was observed in male and female CD-1 mice, with a no adverse effect level (NOAEL) of 10 mg/kg/d (Rodricks et al., 2010) (See, 1996) (Barbolt, 2002; Dayan, 2007). In contrast, TCS was not carcinogenic in a dietary two-year chronic toxicology and carcinogenesis assay in rats. Non-neoplastic lesions reported from the two-year bioassay included a dose-dependent increase in centrilobular hypertrophy particularly in male rats (Source: Goodman DG Pathology Working Group, Ciba) (Rodricks et al., 2010), suggesting induction of cytochrome P450s in the centrilobular (zone 3) region of the liver. No thyroid-related endpoints, including histology or hormone concentrations, were reported for this study, though other chronic exposure studies that examined thyroid histology in multiple species have reported no effects of TCS exposure on thyroid weights or histology (Rodricks et al., 2010).

6d. Reproductive and teratological toxicity

Two generation reproductive studies demonstrated no adverse effects observed in rats or rabbits for reproductive performance, teratology, or postnatal development (Barbolt, 2002; Dayan, 2007); however, the endpoints included in the reproductive toxicology report did not include endocrine or neurological measurements. Thus the NOAEL obtained for rat reproductive toxicology analysis, 50 mg/kg/d for mothers, and a developmental NOAEL for pups as 150-300 mg/kg/d (Bhargava et al., 1996; DeSalva et al., 1989), does not reflect possible developmental thyroid or neuroendocrine effects.

7. Evidence for TCS-induced endocrine disruption and potential mechanisms

Over the last decade a growing body of evidence has emerged to suggest that TCS is an endocrine disruptor in laboratory animals. This includes evidence for anti-androgenic, estrogenic, and anti-thyroid effects. The current work is specifically directed at improving the understanding of thyroid disruption in mammals due to limited suggestive evidence in aquatic models.

7a. Evidence for TCS-induced sex hormone disruption

In vitro receptor-reporter assays suggest that TCS may be weakly anti-estrogenic and possibly weakly anti-androgenic. When TCS was co-applied with 1 nM estradiol in estrogen receptor (ER) reporter assays (CALUX assays), TCS acted as an antagonist, with an IC_{50} of 1 µM TCS (Ahn *et al.*, 2008); however, TCS alone produced no agonist or antagonist effects in this assay (Ahn et al., 2008). Another study using radioligand binding assays demonstrated that large molar excesses of TCS inhibit estradiol binding to ERα, and to a lesser extent ERβ (Gee *et al.*, 2008). Anti-estrogenic effects also were observed in human breast cancer cell lines (MCF-1 and SkBr-3), which suggested that TCS may be an ER antagonist of low

potency (Gee et al., 2008). Yet, in another ER assay using stably transfected T47D human breast cancer cells, TCS was reported as a very weak ER agonist (Houtman *et al.*, 2004).

Potential effects of TCS as an anti-androgen have been reported; TCS inhibited AR-mediated testosterone activity by 92% at 10 µM and by 39% at 1 µM (Chen *et al.*, 2007). In competitive binding assays in mouse mammary and human breast cancer cell lines, TCS antagonized the AR, and inhibited testosterone induction of reporter gene activity (Gee et al., 2008). This web of *in vitro* receptor-reporter data suggests perhaps mixed and weak activity of TCS as a moderate estrogenic and anti-androgenic compound.

Aquatic models of sex hormone disruption further demonstrate a mixed picture of the estrogenic and anti-androgenic action of TCS. A two-week aquatic exposure to developing medaka (*Oryzias latipes*) failed to demonstrate estrogenic effects on the development of phenotypic sex; instead, small changes in fin morphology and non-significant changes to the sex ratio suggested slight androgenic effects (Foran et al., 2000). Strangely a different 21-day study of TCS exposure to medaka demonstrated induction of hepatic vitellogenin in male medaka, suggestive of a weak estrogenic effect, possibly due to a TCS metabolite (Ishibashi et al., 2004). Additionally, anti-estrogenic activity of TCS was observed in aquatic exposures to male South African Clawed frogs (*Xenopus laevis*) (Matsumura et al., 2005).

Recent studies of mammalian *in vivo* sex hormone effects have demonstrated estrogenic effects in rats. In a rat uterotrophic assay, TCS potentially enhanced estrogenic activity; when co-administered with ethinyl estradiol to prepubescent rats, TCS increased uterine weight and histological markers of estrogenic response, compared to TCS exposure alone, which had no effect (Stoker *et al.*, 2010). Zorrilla *et al.* (2009) reported evidence for weak androgenic activity in a study that included evaluation of the histological, morphological, and

hormonal effects of TCS exposure on prepubescent male rats (Zorrilla *et al.*, 2009); a significant decrease in serum testosterone concentration was observed at 200 mg/kg/day, but this was not observed at higher doses and was not dose-dependent (Zorrilla et al., 2009). The current array of *in vitro* and *in vivo* studies on TCS-induced sex hormone disruption appear to support the perspective that TCS may have mild, and potentially mixed, effects on sex hormone status.

7b. Evidence for TCS-induced thyroid hormone disruption

Initial concern for TCS-induced thyroid disruption first arose due to increases in TRβ mRNA expression in *Xenopus laevis* XTC-2 cells following coapplication of TCS and T3 (Veldhoen et al., 2006). In addition, TCS accelerated hind limb formation and decreased weight gain in developing *Rana catesbeina* following T3 treatment-induced metamorphosis (Veldhoen et al., 2006). These results prompted questions regarding the potential for TCS to induce thyroid-related changes in other species. Initial work in our laboratory demonstrated that TCS exposure results in dose-dependent decreases in T4 in weanling female Long-Evans rats in a 4-day exposure model (Crofton et al., 2007). Following this work, other groups have corroborated our finding that TCS exposure decreased T4 in several different rat models (Zorrilla et. al, 2009, Stoker et al 2010, Rodriguez et al 2010).

Further suspicion of potential TCS-induced thyroid hormone disruption stems from *in vitro* evidence for TCS interaction with xenobiotic nuclear receptors. Interaction with the constitutive androstane receptor (CAR) and/or the pregnane X receptor (PXR) is suggestive of potential effects on thyroid hormone homeostasis (Kretschmer *et al.*, 2005; Omiecinski *et al.*, 2011), as CAR and PXR regulate the hepatic enzymes that function in thyroid hormone turnover. Activation of PXR results in transcriptional induction of CYP3A isoforms (Jones

et al., 2000), as well as other Phase I and II hepatic biotransformation enzymes, i.e. glucuronyltransferases and sulfotransferases that catabolize thyroid hormones as part of thyroid hormone homeostasis (Capen, 1994; DeVito *et al.*, 1999; Schuur *et al.*, 1997; Wang *et al.*, 2006). Activation of PXR also transcriptionally regulates Phase III transporters, including organic anion transporting peptides and multidrug resistance proteins (Kretschmer et al., 2005), which are known to mediate hepatic uptake and secretion of thyroid hormones (Jansen *et al.*, 2005). Similary, activation of CAR results in transcriptional up-regulation of CYP2B isoforms, and a set of Phase II and Phase III hepatic enzymes that overlaps with the functional transcriptional activity of PXR (Kretschmer et al., 2005). Thus, activation of CAR and PXR by xenobiotics may lead to increased hepatic catabolic enzyme expression and activity and a subsequent dysregulation of thyroid hormone homeostasis.

A luciferase-based human PXR reporter assay in human hepatoma (HuH7) cells demonstrated moderate *in vitro* activation of PXR by TCS; 10 µM TCS produced approximately 46% of the response of the prototypical inducer rifampicin (Jacobs *et al.*, 2005). Assessment of *in vitro* exposures of rat hepatocytes further support hepatic nuclear receptor activation via demonstrated increases in Cyp2b and Cyp3a1 protein content and enzymatic activity increases in markers of CYP activity including pentoxyresorufin-O-depentylase (PROD), benzyloxyresorufin-O-debentylase (BROD), and ethoxyresorufin-O-deethylase (EROD) (Jinno *et al.*, 1997). *In vivo* TCS exposure also induced hepatic microsomal Cyp2b, Cyp3a, and Cyp4a protein content, as well as PROD and BROD activity in rats (Hanioka *et al.*, 1997). Further, TCS inhibited Cyp1a and Cyp2b induction by 3-methylcholanthrene (3MC) and phenobarbital (PB) treatment, indicating some competitive and/or noncompetitive interaction with the aryl hydrocarbon receptor (AhR) and CAR,

respectively (Hanioka *et al.*, 1996). Evidence of Phase I induction *in vitro* and *in vivo*, in addition to *in vitro* PXR activation by TCS, suggest interactions with xenobiotic nuclear receptors, in particular CAR and/or PXR, as indicated by inductive effects on Cyp2b and Cyp3a respectively. Increased Cyp4a protein (Hanioka et al., 1997) suggests that TCS may also interact with peroxisome proliferator-activated receptor alpha (PPARα) (Johnson *et al.*, 2002c; Johnson *et al.*, 1996; Xu *et al.*, 2005), which is further supported by the induction of PPARα-related liver tumors in mice (Rodricks et al., 2010). As a whole these effects on hepatic xenobiotic nuclear receptors and metabolic enzymes support the findings of TCS-induced changes in THs in rats via activation of nuclear receptors and increased hepatic catabolism.

Discriminating between the potential roles of CAR versus PXR activation by TCS may be difficult due to the promiscuity of these receptors with respect to DNA binding. Both CAR and PXR regulate Cyp3a1 expression in rats by interacting with the DR3 site in the pregnane X response element of CYP3A1 (Smirlis *et al.*, 2001). Similarly, both PXR and CAR regulate Cyp2b, by binding to the DR site within the phenobarbital response element of Cyp2b (Smirlis et al., 2001). Studies in rodents have demonstrated CAR and PXR functional overlap; PXR is capable of binding the phenobarbital responsive enhancer module (PBREM), commonly denoted as the CAR binding site for induction of CYP2B subfamily proteins, and CAR is capable of binding the xenobiotic responsive enhancer module (XREM) commonly denoted as the PXR binding site (Wei *et al.*, 2002). PXR, and CAR to a lesser extent, both have flexible ligand-binding pockets that can accommodate a diverse, species-dependent set of activating compounds (Kretschmer et al., 2005; Omiecinski et al., 2011; Timsit *et al.*, 2007). Understanding whether TCS activates CAR and/or PXR activation may highlight key

events in a potential MOA for TCS-induced hypothyroxinemia that can be tested *in vitro* across species to determine the relevance of data collected in rat models (see Chapter 5).

8. Thyroid hormones regulate physiology

Thyroid hormones modulate a variety of processes in adult and developing organisms in target tissues including brain, pituitary, heart, fat, liver, and bone, from maintenance of metabolic and cardiac output rate to neurodevelopment (Williams, 2008; Yen, 2001; Zoeller et al., 2007a). An important distinction is that disruption of thyroid hormones during neurodevelopment results in permanent alterations, whereas the effects during adulthood are generally reversible with a return to euthyroid levels, naturally or by pharmacological intervention (Miller et al., 2009). The regulation of the thyroid system, the effects of disruption in adults and developing organisms, and potential mechanisms of thyroid disruption are briefly considered below.

8a. Overview of regulation of the thyroid hormone system

Euthyroid status in most vertebrates requires multiple organs and complex regulation maintained by critical negative feedback signals. Regulation begins with the brain: tripeptide thyrotropin-releasing hormone (TRH) is synthesized by paraventricular neurons in the hypothalamus, and transported via vasculature to the anterior pituitary (Zoeller et al., 2007a). This prompts a localized increase in pituitary transcription of thyroid-stimulating hormone (TSH) and post-translational glycosylation to activate this glycoprotein for secretion into the systemic circulation (Yen, 2001; Zoeller et al., 2007a). Upon release of TSH from the pituitary, it binds to surface receptors on the follicular cells of the thyroid gland, initiating a series of events to increase iodothyronine production in the colloid of the follicular cells; bound TSH increases adenylate cyclase, increasing cyclic AMP production, resulting in

increased iodide uptake via the sodium-iodide symporter (NIS), increased thyroglobulin synthesis and iodination, and consequently increased T4 and T3 production and release from the gland (Yen, 2001; Zoeller et al., 2007a). TRH and TSH secretion are under the regulatory negative feedback control of thyroid hormones in circulation (Yen, 2001). Further, hypothalamic concentrations of dopamine and somatostatin may negatively regulate TSH secretion (Yen, 2001), demonstrating layers of negative feedback control at the level of stimulation of iiodothyronine production.

In most vertebrate species, T4 production greatly exceeds T3 production; both iodothyronines are systemically transported to their target tissue sites, but T4 is considered the "prohormone" as T3 mediates the majority of the physiological action of the thyroid via its agonist interaction with the thyroid receptor (TR) (Williams, 2008). T4 is typically bound to serum binding proteins in the circulatory compartment; the ratios and kinetics of these binding proteins is a key difference between thyroid hormone homeostasis between humans and rodent models. Approximately 75% of T4 is bound to thyroxine binding globulin (TBG) in humans (Schussler, 2000); this serum binding protein has high affinity for T4 and a long half-life of 5 days, thereby maintaining the plasma half-life of T4 as 5-9 days in humans (Zoeller et al., 2007a). The remaining T4 pool is bound to transthyretin (TTR) (15%) and albumin (10%) in humans (Schussler, 2000). Both T4 and T3 are actively transported into target tissues (Friesema *et al.*, 1999; Friesema *et al.*, 2005), and T4 is converted to T3 in the periphery by outer ring deiodinases to provide localized increases in availability, providing up to 80% of the T3 in the human body (Chopra *et al.*, 1996; Kohrle, 2002). Thyroid hormones are eliminated from circulation by hepatic catabolism, i.e. by glucuronidation or sulfation (Findlay *et al.*, 2000; Visser *et al.*, 1988; Visser *et al.*, 1990; Yamanaka *et al.*, 2007)

and excreted via the bile. Assays using human jejunum microsomes demonstrated that UGT1A8, UGT1A10, and UGT1A1 catalyze high-affinity glucuronidation of T4 and may result in a lowered absorption rate of T4 from the intestine and increase the excretion of the stable T4 glucuronide via the urine (Yamanaka *et al.*, 2007). Many uncertainties regarding human metabolic clearance and elimination of thyroid hormones from circulation remain due to the absence of comprehensive studies especially in humans.

After reaching target tissues, and conversion of the prohormone T4 to T3, T3 exerts genomic action via interaction with thyroid hormone receptors. TRs are nuclear receptors that act as transcription factors (TFs) with ligand-modulated activity, as the receptor is bound to DNA even in the absence of T3 ligand; this aporeceptor recruits corepressors to inhibit target gene transcription, while T3-activated receptor recruits coactivators through a conformational change, enabling up-regulation of target gene expression (Bernal, 2007; Williams, 2008). Thus, there is a bidirectional regulatory potential, whereby the absence of T3 can repress gene expression and the presence of greater T3 concentrations can result in increased transcription of target genes (Kapoor *et al.*, 2010; Morreale de Escobar *et al.*, 2004). Local cellular availability of T3 to interact with TRs is determined primarily by the outer ring deiodinase 2 (D2) (Gereben *et al.*, 2008). Two subtypes of thyroid receptors (TRs), TRα and TRβ, are expressed on a tissue- and cell-specific basis to regulate localized TH action (Zoeller *et al.*, 2007b); alternative splicing of the transcripts from the genes that encode these subtypes produces three functional receptor isoforms: TRα1, TRβ1, and TRβ2 (Williams, 2008; Yen, 2001). TRβ1 is most highly expressed in liver but present in many tissues, and TRβ2 is most highly expressed in the anterior pituitary (Yen, 2001) and is thought to be a primary determinant of hypothalamic-pituitary-thyroid axis regulation

(Williams, 2008). TRα1 is expressed in neurons (Wallis *et al.*, 2010; Yen, 2001), with levels that are particularly high during fetal development, but expression levels decrease in the weeks following birth to coincide with dramatic increases in TRβ1, suggesting that a developmental pattern of TR isoform expression is related to receptor-specific regulation of genes for neurodevelopment (Yen, 2001). The gene targets of the transcriptional action of TRs in target tissues continue to be elucidated, with lists of TR-regulated genes growing for the brain, heart, and liver (Grijota-Martinez *et al.*, 2011).

In addition to the TR-mediated effects of thyroid hormones, there may be nongenomic actions of thyroid hormones. Thyroid hormone action on angiogenesis and cell proliferation may be mediated by interaction of T3 or T4 with cell surface integrin receptors that initiate signaling through the mitogen-activated protein kinase (MAPK) pathway, resulting in molecular actions including phosphorylation of TRs such that they can autoregulate gene targets (Axelband *et al.*, 2011; Davis *et al.*, 2005; Davis *et al.*, 2008). There is some suggestion that in addition to activation of cellular kinase and calmodulin pathways, T4 alone could affect calcium signaling and transport to modulate cytoskeleton modeling in the cortex of rats (Yen, 2001; Zamoner *et al.*, 2008; Zoeller et al., 2007a). Relatively little is known about the nongenomic action of thyroid hormones in *in vivo* models, but the developing evidence suggests that there may be important TR-independent roles for the "prohormone" T4 in addition to T3.

8b. Physiological action of thyroid hormones in adult organisms

The function of thyroid hormones in adult organisms is quite different from the function of thyroid hormones in the developing neonate. In adults, thyroid hormones modulate cardiac output, metabolism, nervous system control of mental and physical ability, including

muscle tone, calcium-phosphate balance for bone density, pituitary and adrenocortical function, respiration, and reproductive function (Yen, 2001). There is further evidence that TRs and TR mutations may be involved in the development of human cancers in myriad tissues (Cheng, 2003; Gonzalez-Sancho *et al.*, 2003). Autoimmune diseases of the thyroid are the largest cause of dysregulation of thyroid hormone function in adult humans. Grave's disease and Hashimoto's thyroiditis demonstrate a prevalence of 5-10% in the population, and result in hyperthyroidism and loss of thyroid function, respectively (Michels *et al.*, 2010). Fortunately the clear health risks exerted by dysregulation of TH concentrations are reversible with T4 supplementation, or treatment with antithyroid drugs, radioablation of the thyroid, or surgical removal of the thyroid followed by T4 supplementation (Arbelle *et al.*, 1999).

8c. Thyroid hormones during development

In contrast to adult humans, thyroid dysregulation during development has deleterious and permanent adverse consequences on the developing organism. Severe thyroid hormone deficiencies during the first trimester of pregnancy result in neurological cretinism, including motor skill deficits and mental retardation, and if deficiency is continued through the second trimester, the cerebral cortex, basal ganglia, and cochlea are susceptible to irreversible injury (Berbel *et al.*, 2007; Bernal, 2002; Williams, 2008). Generally, there are three phases of the thyroid hormone environment during neurodevelopment: the first trimester period during which maternal thyroid hormones are the only source of thyroid hormones; at 16-20 weeks post-conception in humans and embryonic day 17.5-18 in rats, fetal thyroid function begins to complement maternal supplies of thyroid hormone; and, finally, the early postnatal period during which neonatal thyroid hormones alone modulate remaining neurodevelopment

(Morreale de Escobar *et al.*, 2000; Williams, 2008). Available T3 in the developing brain activates TRs to transcriptionally regulate required gene expression for a host of neurodevelopmental processes including myelination, neuronal and glial cell differentiation and migration during and pregnancy and the early postnatal period (Bernal, 2007). Prior to the onset of fetal thyroid function, neurogenesis and neuronal migration are regulated by maternal T4 (Auso *et al.*, 2004; Cuevas *et al.*, 2005; Lavado-Autric et al., 2003). Following the onset fetal thyroid function, maternal thyroid hormones still supplement fetal thyroid hormone production to regulate neurogenesis, neuronal migration, axonal outgrowth, dendritic branching, synaptogenesis, glial cell development, and the beginnings of myelination; these susceptible ongoing, thyroid hormone-modulated processes can be adversely impacted by disruption of maternal T4 supply (de Escobar *et al.*, 2008; Morreale de Escobar et al., 2000; Morreale de Escobar et al., 2004). Postnatally, neuronal cell migration in the hippocampus and cerebellum continue along with glial cell development and myelination, requiring normal neonatal thyroid function (Porterfield *et al.*, 1993; Williams, 2008). All of these processes are subject to permanent alteration in the event that thyroid hormone signaling is disrupted.

The correlation between maternal hypothyroxinemia and irreversible neonatal cognitive and brain abnormalities is well established (Barone *et al.*, 2000; Berbel *et al.*, 2009; Cuevas et al., 2005; Howdeshell, 2002; Morreale de Escobar et al., 2000; Rice *et al.*, 2000; Zoeller et al., 2000). Even relatively small changes in maternal T4 during pregnancy affect the IQ of human children (Haddow *et al.*, 1999) and other measures of cognition, socialization, and motor function (Berbel et al., 2009; Kooistra et al., 2006; Li *et al.*, 2010; Pop et al., 2003; Pop et al., 1999). The lasting effects of decreased *in utero* T4 may be explained by rodent

models which demonstrate correlations between decreased maternal T4 and alterations in cytoarchitecture (Auso et al., 2004; Cuevas et al., 2005; Lavado-Autric et al., 2003; Sharlin *et al.*, 2008), decreased expression of proteins involved in calcium regulation at the synapse and myelination (Ibarrola *et al.*, 1997; Iniguez *et al.*, 1996), and/or changes in the expression of genes associated with calcium homeostasis at the synapse, transcriptional regulation, synapse transmission, myelination and developmental cell adhesion (de Escobar et al., 2008; Morreale de Escobar et al., 2000; Morreale de Escobar et al., 2004; Royland *et al.*, 2008). Maternal production of both T4 and T3 increase during normal pregnancy, with a sharp spike in serum T4 concentrations during the first trimester (Glinoer, 1997), corresponding to increased disposition of maternal T4 to the fetal compartment (Morreale de Escobar et al., 2004). In the fetal compartment during early gestation, T4 is found predominantly as fT4, and is dependent upon the T4 and fT4 in the systemic circulation of the mother; thus, subclinical maternal hypothyroxinemia may affect the thyroid status of the developing progeny (de Escobar *et al.*, 2008). Disruption of maternal T4, even in the absence of overt hypothyroidism, classically defined as decreased T4 and increased TSH, may result in adverse effects on fetal neurodevelopment.

8d. Mechanisms of thyroid hormone disruption

Thyroid-disrupting chemicals include xenobiotics that alter structure or function of the thyroid gland itself, interfere with regulatory enzymatic mechanisms responsible for thyroid hormone homeostasis, or change systemic or tissue thyroid hormone concentrations (Capen, 1994; Crofton et al., 2005a; DeVito et al., 1999). Thyroid disruptors act via multiple targets, including: inhibition of thyroid hormone synthesis by disruption of iodide uptake or thyroperoxidase; binding to thyroid hormone transport proteins; interference with TRs

systemically; inhibition of iodothyronine deiodinases at sites of thyroid action; and increased rates of clearance of THs (Boas *et al.*, 2006; Capen, 1994; Crofton, 2008).

Prevention of iodothyronine production at the level of the thyroid gland has been demonstrated by toxicants that inhibit iodide uptake via the sodium-iodide symporter (NIS) or catalysis of iodide to iodine for conjugation to tyrosine residues on thyroglobulin by thyroperoxidase (TPO) (Crofton, 2008). Perchlorate (Wolff, 1998), nitrate, and thiocyanate (De Groef *et al.*, 2006) are known environmental contaminants that inhibit NIS function. The antithyroid drugs propylthiouracil (PTU) and methimazole (MMI) inhibit TPO (Cooper, 2005), as well as the ultraviolet filter benzophenone-2 (Schmutzler *et al.*, 2007) and isoflavones (Chang *et al.*, 2000) among others (Crofton, 2008).

Many thyroid disrupting chemicals act extrathyroidally via: agonism or antagonism of TRs; displacement of thyroid hormones from serum binding proteins; and, disruption of normal rats of thyroid hormone metabolism. Few xenobiotics have been reported to interact with TRs; the environmental contaminant bisphenol-A is an antagonist *in vitro* (Moriyama *et al.*, 2002; Zoeller *et al.*, 2005), and Aroclor 1254, a mixture of polychlorinated biphenyls (PCBs), appeared to exert agonist action against TR *in vivo* (Gauger *et al.*, 2007; Gauger *et al.*, 2004), although the neuroendocrine response to Aroclor 1254 was not fully attributable to unidirectional agonism (Bansal *et al.*, 2008). Xenobiotic competition for transthyretin (TTR), a minor thyroid hormone binding protein in humans, has been suggested as an alternate mode of thyroid hormone disruption by toxicants like PCBs (Lans *et al.*, 1994; van den Berg, 1990). However, this MOA has been disputed for PCBs by *in vivo* studies in rats that indicated that the timing of peak serum concentrations of PCBs did not result in increased fT4 in serum (Hedge *et al.*, 2009).

A widely accepted MOA for thyroid hormone disruption is up-regulation of thyroid hormone metabolism, either by inhibition of peripheral deiodination of T4 to T3 (Kohrle, 2002; Kohrle, 1996) or up-regulation of hepatic catabolism and transport of thyroid hormones (Barter *et al.*, 1994; Capen, 1994; Hood *et al.*, 2003; Johnson *et al.*, 2002b; Klaassen *et al.*, 2005; Lecureux *et al.*, 2009; Vansell *et al.*, 2001). Xenobiotics including PCBs (Morse *et al.*, 1993), PTU (Visser *et al.*, 1979), and FD&C Red No. 3 dye (Capen, 1998) have been implicated in inhibition of peripheral deiodination. Many thyroid disrupting chemicals have been proposed to activate xenobiotic nuclear receptors including the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR) to increase metabolism and elimination of thyroid hormones. AhR, CAR, and PXR transcriptionally regulate gene expression of glucuronyltransferase, sulfotransferase, and hepatic transporter isoforms necessary for catabolism and elimination of thyroid hormones at a normal rate to maintain euthyroid status (Craft et al., 2002; Crofton et al., 2005a; Kohn, 2000; Kretschmer et al., 2005; Timsit *et al.*, 2007). Activation of these receptors by xenobiotics has resulted in up-regulation of glucuronidation, sulfation, and export of thyroid hormones leading to thyroid hormone decreases by a variety of compounds, including examples such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methycholanthrene (3-MC), Aroclor 1254, PCBs, phenobarbital (PB), pregnenolone-16alpha-carbonitrile (PCN), DMP-904, and oltipraz that have largely been described in rodent and *in vitro* human models as microsomal enzyme inducers (Barter et al., 1994; Buckley *et al.*, 2009; Cheng *et al.*, 2005; Guo *et al.*, 2002; Johnson et al., 2002b; Kohn, 2000; Lecureux et al., 2009; Schuur et al., 1997; Vansell *et al.*, 2002; Vansell et al., 2001; Visser et al., 1993; Wong *et al.*, 2005). The basic hypothesis is that these microsomal enzyme

inducers cause a systemic thyroid hormone concentration decrease because more thyroid hormone exits the system following increased metabolic clearance and/or transport. Recently the importance of increased T4 glucuronidation by the Ugt1a subfamily in mediating serum T4 decreases has been questioned by studies in the rat, which demonstrated that T4 decreases were possible in Ugt1a-deficient Gunn rats following microsomal enzyme induction with Aroclor 1254, 3-MC, and PCN (Richardson *et al.*, 2010). Previously a poor correlation between T4-glucuronidation and T4 decreases and TSH increases has been observed (Craft et al., 2002; Hood *et al.*, 2000; Vansell et al., 2001), and the decrease in serum T4 observed with PB treatment in Gunn rats also demonstrated a lack of dependence on Ugt1a-mediated glucuronidation of T4 (Kato *et al.*, 2005). These data do not negate the potential contribution of T4-glucuronidation to observed systemic decreases in T4, but rather suggest that other mechanisms, identified or yet to be identified, contribute to decreased thyroid hormone concentrations. One possibility is the contribution of thyroid hormone sulfation. Another speculation might be that increased clearance of thyroid hormones may rely on increased export of parent T4, as observed with DMP-904 in rats (Wong et al., 2005). Hepatic catabolic modulation of thyroid hormone homeostasis and characterization of the associated MOA require further clarification.

9. Rat as a model of TH-disruption

Particularly for environmental toxicants, it is difficult to obtain human data; *in vivo* human data for thyroid hormone disruptors is limited and centers on pharmacological agents with off-target thyroid toxicity or epidemiological studies burdened by weak exposure metrics. Fortunately, rats appropriately model the neurological effects of developmental thyroid hormone insufficiency (Zoeller *et al.*, 2004). The general mechanisms of thyroid

hormone homeostasis are conserved, but key differences in the specific gene and protein isoforms expressed do instill uncertainty to extrapolation across species. Some of the potential differences between rat and human thyroid hormone homeostasis mechanisms are described here, as well as the potential human plausibility of the MOA described in rat and *in vitro* models.

9a. Potential relevance to humans: differences in TH homeostasis between rats and humans

Thyroid hormones are maintained in circulation primarily bound to serum binding proteins and are cleared from the system at some constant rate as a result of hepatic catabolism and excretion. There are substantial differences in the relative expression of serum binding proteins and the half-life of T4 between humans and rats. In contrast to humans, thyroxine-binding globulin (TBG) is only expressed during development and early postnatal life in rats (Savu *et al.*, 1987; Vranckx *et al.*, 1994; Vranckx *et al.*, 1990), and following this period the primary serum binding protein is transthyretin (TTR) (Savu et al., 1987; Vranckx et al., 1994). TTR has a lower binding affinity and reduced half-life compared to TBG, resulting in a shortened plasma half-life for rat T4 that is 12-24 hours instead of 5-9 days in humans. This decreased T4 half-life in rats may make them more susceptible to thyroid disruption than humans (Capen, 1994).

The inducibility of TSH also varies between rats and humans. Decreased thyroid hormones in circulation initiates an up-regulation of TSH in rodents that can excessively stimulate the thyroid and result in thyroid tumors (Hill *et al.*, 1998). Humans are thought to be much less sensitive to this MOA due to a larger circulating pool of THs and a larger luminal supply of THs in the thyroid gland (Capen, 1994; Hill *et al.*, 1998).

An additional uncertainty in using rats to model human TH homeostasis is enterohepatic recirculation of thyroid hormones, which may change the relative excretion pathway and rates between species. The hypothesis we explore in this work is that TCS up-regulates hepatic catabolism and excretion of thyroid hormones; however, it is possible that some fraction, potentially divergent between species, is recirculated rather than exiting via biliary excretion. It is fairly well accepted that rats conjugate thyroid hormones largely by glucuronidation in preparation for a large fraction to be fecally excreted via the bile (Beetstra *et al.*, 1991; de Herder *et al.*, 1988; Kaptein *et al.*, 1997; Visser, 1996; Visser *et al.*, 1993; Visser et al., 1990). *In vivo* rat studies have demonstrated that bile recirculates to the intestine, where hydrolysis of thyroid hormone and predominantly T4-glucuronide (Visser *et al.*, 1990) may enable as much as 50% of thyroid hormone entering the intestine to be reabsorbed, with the rest eliminated fecally (DiStefano *et al.*, 1988). In contrast to the findings of DiStefano and Visser, Galton and Nisula (1972) found no net resorption of thyroid hormone from the gastro-intestinal tract of rats (Galton *et al.*, 1972). A separate study confirmed that the majority of thyroid hormone conjugate excreted into bile is T4-glucuronide, and that though this conjugate is enterally recirculated, this conjugate has a high affinity for bile, with very little actually re-entering systemic circulation (Bastomsky, 1972). Limited evidence from human patients suggests that enterohepatic recirculation of thyroid hormone and its conjugates occurs, but that only 30% is reabsorbed in the intestine, with 70% of the thyroid hormone pool in the bile exiting fecally (Myant, 1956). Differences in reabsorption of thyroid hormone in the intestine may constitute a kinetic difference in thyroid hormone maintenance between rats and humans. However, comparison between species is difficult due to the limited number of studies particularly for humans.

9b. Potential relevance to humans: evidence for thyroid disruption in humans by microsomal enzyme inducers

Increased hepatic catabolism and elimination of thyroid hormones via microsomal enzyme induction is one MOA for thyroid hormone disruption that has been increasingly noted in rodent models of thyroid toxicology (Barter et al., 1994; Brucker-Davis, 1998; Guo et al., 2002; Hood et al., 2003; Hood *et al.*, 1999; Hood et al., 2000; Hurley, 1998; Johnson *et al.*, 2002a; Lecureux et al., 2009; Liu *et al.*, 1995; Vansell et al., 2001). This MOA is potentially relevant to humans because similar glucuronidation and sulfation catabolic pathways also lead to increased thyroid hormone turnover. There are also analagous xenobiotic nuclear receptors in rats and humans that regulate similar functional domains to include Phase I, Phase II, and hepatic transport expression.

In vivo evidence for the plausibility of the hypothesis that thyroid disrupting chemicals can decrease serum thyroid hormones in humans comes primarily from the toxic side-effects of therapeutic drugs. A number of drugs, including lithium (anti-depressant) (Gittoes *et al.*, 1995) and the anti-convulsant sedatives carbamazepine, phenytoin, and phenobarbital, are all known to decrease thyroid hormones (Gittoes et al., 1995; Simko *et al.*, 2007). Carbamazepine decreased systemic T4 via activation of hepatic microsomal enzymes and displacement and also potentially by competitive binding to TBG (Simko et al., 2007); carbamazepine induces CYP3A4 and UGT1A1 (Oscarson *et al.*, 2006), and is a PXR agonist (Luo *et al.*, 2002), suggesting that it can up-regulate glucuronidation and other catabolic processes to increase elimination of thyroxine. Phenobarbital is also thought to increase glucuronidation of T4 via activation of CAR, and increases expression and activity of UGT1A1 in human hepatocytes (Ritter *et al.*, 1999), though at concentrations that exceed

therapeutic serum concentrations by at least 10-fold (Strolin et al 2005). In addition to increased glucuronidation activity, phenytoin may increase peripheral conversion of T4 to T3 (Strolin et al 2005). The therapeutic use of anti-convulsant sedatives appears to mediate moderate inductive effects on metabolic enzymes and the potential for thyroid hormone disruption (Strolin et al. 2005, (Perucca *et al.*, 1984; Simko et al., 2007). On this basis, it appears possible to elevate glucuronidation activity to coincide with serum T4 decreases in humans, provided a high enough dose of the microsomal enzyme inducer is employed.

Several epidemiological studies have tried to retrospectively correlate the serum concentrations of known thyroid disrupting toxicants with serum thyroid hormone concentrations, with varying success. Chevrier *et al.* (2010) found an inverse correlation between the serum concentration of polybrominated diphenyl ethers (PBDEs) and maternal serum TSH, which does not correspond to the expected relationship that PBDEs decrease serum T4 and T3 concentrations with no effects on TSH as seen in rodents (Zhou *et al.*, 2002). An inverse correlation between the serum concentrations of hydroxylated PCBs in mothers and children and markers of neurodevelopment was demonstrated in a cohort from Slovakia (Park *et al.*, 2009). Similar reports are counterbalanced by reports that demonstrate little or no correlation between thyroid hormone disruptor burden in maternal and/or child tissues and neurodevelopment and thyroid hormone concentrations in the children (Darnerud *et al.*, 2010). Thus, there is some suggestion that body burden of thyroid disrupting compounds may correspond to thyroid hormone and/or neurological outcomes, but these associations are weakly correlative at best, likely due, in part, to the epidemiological experimental approach and confounding factors.

10. Summary and human health significance

Currently, there is a veritable dearth of evidence regarding the potential risks to human health posed by widespread TCS environmental contamination. Detection of TCS in ecosystems and human tissue samples globally creates an imperative to determine the dose-response relationship for thyroid hormone disruption by TCS, the MOA of TCS-induced hypothyroxinemia, and the potential effects of TCS on the developing neonate. TCS may interact with hepatic xenobiotic nuclear receptors, specifically CAR and/or PXR, and produce downstream hepatic enzyme induction that may alter thyroid hormone homeostasis. Currently, there is a lack of information regarding the MOA for TCS-induced hypothyroxinemia in the rat. Knowledge of the MOA in *in vivo* laboratory models is extremely useful for determination of the human relevance of TCS-induced hypothyroxinemia (Boobis *et al.*, 2008; Crofton *et al.*, 2005b). Common hazard assessment practice assumes human relevance of animal data; analysis of the key events of the MOA in rat models and *in vitro* human models enables testing of the hypothesis that TCS-induced hypothyroxinemia is relevant to humans.

11. Experimental Design

In vivo oral administration of TCS to rats is used in this research in order to model human oral exposures that would potentially occur through drinking water or oral hygiene products. In addition, *in vitro* experiments were used to address specific mechanistic questions regarding the potential activation of xenobiotic nuclear receptors by TCS and the contribution of functional hepatic transport to the hypothesized MOA. *In vivo*, whole animal exposures were used to identify the target tissue; the liver was identified as critically involved in mediating the observed systemic thyroid hormone disruption. Initially a short-

term exposure of four days was employed to assess the potential for TCS to decrease thyroid hormones in young female rats, as a screen for thyroid hormone disruption, and to identify an appropriate dose range for further characterization of the MOA for TCS-induced thyroid hormone disruption in rats. A developmental exposure paradigm was used to model the potential exposure and effects of TCS on the developing organism; rat dams were exposed perinatally to TCS via oral gavage, such that offspring only received TCS through transplacental transfer and/or milk consumption. This was a more appropriate model for the subpopulation of concern: human neonates receiving TCS via transplacental transfer and lactation. Finally, the human relevance of the *in vivo* rat work was assessed by comparative *in vitro* models of the initiating key event of the MOA hypothesis: xenobiotic nuclear receptor activation, as indicated by species-specific receptor-reporter assays.

The second chapter of this work describes the effects of a four-day TCS exposure on the thyroid hormone system and biomarkers of hepatic catabolism of thyroid hormones. Hepatic microsomal activity assays and RT-PCR were used to assess the contribution of hepatic catabolism, i.e. glucuronidation and sulfation, and transport, to the observed systemic decreases in thyroid hormones. The third chapter of this work details study of the susceptible population model: the developing fetus and neonate. This work provided an initial focus on the potential for thyroid hormone disruption during the lactation period. The fourth chapter includes an extension of the initial finding that TCS decreases thyroxine (T4) during postnatal development to include assessment of fetal thyroxine during gestation, an examination of the hypothesized MOA in multiple life-stages, and an assessment of the internal doses of TCS received at the various life-stages. Receptor-reporter assays indicating xenobiotic nuclear receptor activation, and providing critical interspecies comparison data

between rat and humans, are described in the fifth chapter of this work. The sixth chapter of this work summarizes the major findings of the research project, implications, and future directions of this work. Finally, an appendix details the effects of TCS on *in vitro* functional hepatic transport in sandwich-cultured rat hepatocytes as an addendum to Specific Aim 1.

Global hypothesis: TCS induces hypothyroxinemia via activation of xenobiotic nuclear receptors, resulting in up-regulation of hepatic catabolism and excretion of thyroid hormones and subsequent systemic thyroid hormone concentration decreases.

Hypothesis 1: Short-term TCS exposure decreases T4 with coincident up-regulation of markers of hepatic catabolism and transport of thyroid hormones, suggesting that the MOA of TCS-induced hypothyroxinemia is increased hepatic catabolism and excretion of T4.

Specific Aim 1: Determine whether triclosan affects hepatic catabolism and transport of thyroxine in rats. Assessment of hepatic Phase I and Phase II gene expression and enzymatic markers reflective of nuclear receptor activation was performed using quantitative reverse transcriptase PCR (qRT-PCR) and hepatic microsomal assays. The potential contribution of hepatic transport to the proposed MOA was measured by qRT-PCR of key hepatic transport proteins (Phase III). Follow-up studies of hepatic transport function were performed in an additional set of *in vitro* experiments in sandwich-cultured rat hepatocytes.

Hypothesis 2: Perinatal TCS exposure decreases T4 in dams and offspring via a similar proposed MOA: increased hepatic catabolism and elimination of thyroid hormones as a result of xenobiotic nuclear receptor activation by TCS.

Specific Aim 2: Determine the effect of perinatal maternal exposure on thyroxine concentrations and metabolism in dams and offspring. The dose-response relationship for

perinatal maternal TCS exposure and T4 decreases was determined in dams and pups; analysis of TCS in the serum and liver of exposed animals provided additional support for these relationships. Hepatic microsomal activity assays were employed to study markers of Phase I and II metabolic enzyme activity, and the mRNA expression of Phase I, II, and III hepatic metabolic enzymes was evaluated using quantitative PCR (qPCR). Gene expression markers of tissue-level thyroid hormone changes were assessed for hepatic mRNA by qPCR.

Hypothesis 3: TCS activates rat and human xenobiotic nuclear receptors as the initiating key event in a MOA for TCS-induced hypothyroxinemia.

Specific Aim 3: Determine whether TCS activates the constitutive androstane and pregnane X receptors (CAR and PXR) in models of the human and rat receptors. The *in vitro* activation of human and rat PXR was measured using commercially available (Puracyp, Inc.; Carlsbad, CA) human and rat hepatoma cell lines containing full-length PXR and a luciferase-based reporter construct. Cell-based receptor-reporter assays in human embryonic kidney cells (HEK293T) (INDIGO BioSciences) were used to evaluate the ligand-binding potential of TCS for all three active human CAR splice variants (hCAR1, hCAR2, hCAR3), rat CAR, mouse CAR, and human, rat, and mouse PXR.

References

Adolfsson-Erici, M., M. Pettersson, J. Parkkonen and J. Sturve (2002). Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**, 1485-1489.

Ahn, K. C., B. Zhao, J. Chen, G. Cherednichenko, E. Sanmarti, M. S. Denison, B. Lasley, I. N. Pessah, D. Kultz, D. P. Chang, S. J. Gee and B. D. Hammock (2008). In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. *Environmental health perspectives* **116**, 1203-1210.

Aiello, A. E., E. L. Larson and S. B. Levy (2007). Consumer antibacterial soaps: effective or just risky? *Clin Infect Dis* **45 Suppl 2**, S137-147.

Allmyr, M., M. Adolfsson-Erici, M. S. McLachlan and G. Sandborgh-Englund (2006a). Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *The Science of the total environment* **372**, 87-93.

Allmyr, M., M. S. McLachlan, G. Sandborgh-Englund and M. Adolfsson-Erici (2006b). Determination of triclosan as its pentafluorobenzoyl ester in human plasma and milk using electron capture negative ionization mass spectrometry. *Analytical chemistry* **78**, 6542-6546.

Allmyr, M., F. Harden, L. M. Toms, J. F. Mueller, M. S. McLachlan, M. Adolfsson-Erici and G. Sandborgh-Englund (2008). The influence of age and gender on triclosan concentrations in Australian human blood serum. *The Science of the total environment* **393**, 162-167.

Allmyr, M., G. Panagiotidis, E. Sparve, U. Diczfalusy and G. Sandborgh-Englund (2009). Human exposure to triclosan via toothpaste does not change CYP3A4 activity or plasma concentrations of thyroid hormones. *Basic Clin Pharmacol Toxicol* **105**, 339-344.

Arbelle, J. E. and A. Porath (1999). Practice guidelines for the detection and management of thyroid dysfunction. A comparative review of the recommendations. *Clinical endocrinology* **51**, 11-18.

Auso, E., R. Lavado-Autric, E. Cuevas, F. E. Del Rey, G. Morreale De Escobar and P. Berbel (2004). A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration. *Endocrinology* **145**, 4037-4047.

Axelband, F., J. Dias, F. M. Ferrao and M. Einicker-Lamas (2011). Nongenomic signaling pathways triggered by thyroid hormones and their metabolite 3-iodothyronamine on the cardiovascular system. *J Cell Physiol* **226**, 21-28.

Bagley, D. M. and Y. J. Lin (2000). Clinical evidence for the lack of triclosan accumulation from daily use in dentifrices. *American journal of dentistry* **13**, 148-152.

Balmer, M. E., T. Poiger, C. Droz, K. Romanin, P. A. Bergqvist, M. D. Muller and H. R. Buser (2004). Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland. *Environmental science & technology* **38**, 390-395.

Bansal, R. and R. T. Zoeller (2008). Polychlorinated biphenyls (Aroclor 1254) do not uniformly produce agonist actions on thyroid hormone responses in the developing rat brain. *Endocrinology* **149**, 4001-4008.

Barbolt, T. A. (2002). Chemistry and safety of triclosan, and its use as an antimicrobial coating on Coated VICRYL* Plus Antibacterial Suture (coated polyglactin 910 suture with triclosan). *Surgical infections* **3 Suppl 1**, S45-53.

Barone, S., Jr., K. P. Das, T. L. Lassiter and L. D. White (2000). Vulnerable processes of nervous system development: a review of markers and methods. *Neurotoxicology* **21**, 15-36.

Barter, R. A. and C. D. Klaassen (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. *Toxicology and applied pharmacology* **128**, 9-17.

Bastomsky, C. H. (1972). The enterohepatic circulation of thyroxine in the rat: a short circuit. *Clin Sci* **42**, 28P-29P.

Beetstra, J. B., J. G. van Engelen, P. Karels, H. J. van der Hoek, M. de Jong, R. Docter, E. P. Krenning, G. Hennemann, A. Brouwer and T. J. Visser (1991). Thyroxine and 3,3',5-triiodothyronine are glucuronidated in rat liver by different uridine diphosphate-glucuronyltransferases. *Endocrinology* **128**, 741-746.

Berbel, P., M. J. Obregon, J. Bernal, F. Escobar del Rey and G. Morreale de Escobar (2007). Iodine supplementation during pregnancy: a public health challenge. *Trends Endocrinol Metab* **18**, 338-343.

Berbel, P., J. L. Mestre, A. Santamaria, I. Palazon, A. Franco, M. Graells, A. Gonzalez-Torga and G. M. de Escobar (2009). Delayed neurobehavioral development in children born to pregnant women with mild hypothyroxinemia during the first month of gestation: the importance of early iodine supplementation. *Thyroid* **19**, 511-519.

Berbel, P., D. Navarro, E. Auso, E. Varea, A. E. Rodriguez, J. J. Ballesta, M. Salinas, E. Flores, C. C. Faura and G. M. de Escobar (2010). Role of late maternal thyroid hormones in cerebral cortex development: an experimental model for human prematurity. *Cereb Cortex* **20**, 1462-1475.

Bernal, J. (2002). Action of thyroid hormone in brain. *J Endocrinol Invest* **25**, 268-288.

Bernal, J. (2007). Thyroid hormone receptors in brain development and function. *Nat Clin Pract Endocrinol Metab* **3**, 249-259.

Bhargava, H. N. and P. A. Leonard (1996). Triclosan: applications and safety. *American journal of infection control* **24**, 209-218.

Black, J. G., D. Howes and T. Rutherford (1975). Percutaneous absorption and metabolism of Irgasan DP300. *Toxicology* **3**, 33-47.

Boas, M., U. Feldt-Rasmussen, N. E. Skakkebaek and K. M. Main (2006). Environmental chemicals and thyroid function. *European journal of endocrinology / European Federation of Endocrine Societies* **154**, 599-611.

Bock, M., J. Lyndall, T. Barber, P. Fuchsman, E. Perruchon and M. Capdevielle (2010). Probabilistic application of a fugacity model to predict triclosan fate during wastewater treatment. *Integr Environ Assess Manag* **6**, 393-404.

Boobis, A. R., J. E. Doe, B. Heinrich-Hirsch, M. E. Meek, S. Munn, M. Ruchirawat, J. Schlatter, J. Seed and C. Vickers (2008). IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Critical reviews in toxicology* **38**, 87-96.

Brucker-Davis, F. (1998). Effects of environmental synthetic chemicals on thyroid function. *Thyroid* **8**, 827-856.

Buckley, D. B. and C. D. Klaassen (2009). Induction of mouse UDP-glucuronosyltransferase mRNA expression in liver and intestine by activators of aryl-hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, peroxisome proliferator-activated receptor alpha, and nuclear factor erythroid 2-related factor 2. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 847-856.

Butte, N. F., C. Garza, E. O. Smith and B. L. Nichols (1984). Human milk intake and growth in exclusively breast-fed infants. *J Pediatr* **104**, 187-195.

Butte, N. F., M. G. Lopez-Alarcon and C. Garza (2002). Nutrient adequacy of exclusive breast feeding for the term infant during the first 6 months of life. *WHO* **Geneva**, 1-49.

Calafat, A. M., X. Ye, L. Y. Wong, J. A. Reidy and L. L. Needham (2008). Urinary concentrations of triclosan in the U.S. population: 2003-2004. *Environmental health perspectives* **116**, 303-307.

Capen, C. C. (1994). Mechanisms of chemical injury of thyroid gland. *Progress in clinical and biological research* **387**, 173-191.

Capen, C. C. (1998). Correlation of mechanistic data and histopathology in the evaluation of selected toxic endpoints of the endocrine system. *Toxicology letters* **102-103**, 405-409.

Chalew, T. E. and R. U. Halden (2009). Environmental Exposure of Aquatic and Terrestrial Biota to Triclosan and Triclocarban. *J Am Water Works Assoc* **45**, 4-13.

Chang, H. C. and D. R. Doerge (2000). Dietary genistein inactivates rat thyroid peroxidase in vivo without an apparent hypothyroid effect. *Toxicology and applied pharmacology* **168**, 244-252.

Chen, J., K. C. Ahn, N. A. Gee, S. J. Gee, B. D. Hammock and B. L. Lasley (2007). Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. *Toxicology and applied pharmacology* **221**, 278-284.

Cheng, S. Y. (2003). Thyroid hormone receptor mutations in cancer. *Mol Cell Endocrinol* **213**, 23-30.

Cheng, X., J. Maher, M. Z. Dieter and C. D. Klaassen (2005). Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1276-1282.

Chopra, I. J., P. Taing and L. Mikus (1996). Direct determination of free triiodothyronine (T3) in undiluted serum by equilibrium dialysis/radioimmunoassay (RIA). *Thyroid* **6**, 255-259.

Coogan, M. A., R. E. Edziyie, T. W. La Point and B. J. Venables (2007). Algal bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater treatment plant receiving stream. *Chemosphere* **67**, 1911-1918.

Cooper, D. S. (2005). Antithyroid drugs. *The New England journal of medicine* **352**, 905-917.

Craft, E. S., M. J. DeVito and K. M. Crofton (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**, 372-380.

Crofton, K. M., E. S. Craft, J. M. Hedge, C. Gennings, J. E. Simmons, R. A. Carchman, W. H. Carter, Jr. and M. J. DeVito (2005a). Thyroid-hormone-disrupting chemicals: evidence for dose-dependent additivity or synergism. *Environmental health perspectives* **113**, 1549-1554.

Crofton, K. M. and R. T. Zoeller (2005b). Mode of action: neurotoxicity induced by thyroid hormone disruption during development--hearing loss resulting from exposure to PHAHs. *Critical reviews in toxicology* **35**, 757-769.

Crofton, K. M., K. B. Paul, J. M. Hedge and M. J. DeVito (2007). Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine. *Environ Toxicol Pharmacol* **24**, 194-197.

Crofton, K. M. (2008). Thyroid disrupting chemicals: mechanisms and mixtures. *International journal of andrology* **31**, 209-223.

Cuevas, E., E. Auso, M. Telefont, G. Morreale de Escobar, C. Sotelo and P. Berbel (2005). Transient maternal hypothyroxinemia at onset of corticogenesis alters tangential migration of medial ganglionic eminence-derived neurons. *Eur J Neurosci* **22**, 541-551.

Darnerud, P. O., S. Lignell, A. Glynn, M. Aune, A. Tornkvist and M. Stridsberg (2010). POP levels in breast milk and maternal serum and thyroid hormone levels in mother-child pairs from Uppsala, Sweden. *Environment international* **36**, 180-187.

Davis, P. J., F. B. Davis and V. Cody (2005). Membrane receptors mediating thyroid hormone action. *Trends Endocrinol Metab* **16**, 429-435.

Davis, P. J., J. L. Leonard and F. B. Davis (2008). Mechanisms of nongenomic actions of thyroid hormone. *Front Neuroendocrinol* **29**, 211-218.

Dayan, A. D. (2007). Risk assessment of triclosan [Irgasan((R))] in human breast milk. *Food Chem Toxicol* **45**, 125-129.

de Escobar, G. M., S. Ares, P. Berbel, M. J. Obregon and F. E. del Rey (2008). The changing role of maternal thyroid hormone in fetal brain development. *Semin Perinatol* **32**, 380-386.

De Groef, B., B. R. Decallonne, S. Van der Geyten, V. M. Darras and R. Bouillon (2006). Perchlorate versus other environmental sodium/iodide symporter inhibitors: potential thyroid-related health effects. *European journal of endocrinology / European Federation of Endocrine Societies* **155**, 17-25.

de Herder, W. W., F. Bonthuis, M. Rutgers, M. H. Otten, M. P. Hazenberg and T. J. Visser (1988). Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. *Endocrinology* **122**, 153-157.

DeSalva, S. J., B. M. Kong and Y. J. Lin (1989). Triclosan: a safety profile. *American journal of dentistry* **2 Spec No**, 185-196.

DeVito, M., L. Biegel, A. Brouwer, S. Brown, F. Brucker-Davis, A. O. Cheek, R. Christensen, T. Colborn, P. Cooke, J. Crissman, K. Crofton, D. Doerge, E. Gray, P. Hauser, P. Hurley, M. Kohn, J. Lazar, S. McMaster, M. McClain, E. McConnell, C. Meier, R. Miller, J. Tietge and R. Tyl (1999). Screening methods for thyroid hormone disruptors. *Environmental health perspectives* **107**, 407-415.

DiStefano, J. J., 3rd, M. Sternlicht and D. R. Harris (1988). Rat enterohepatic circulation and intestinal distribution of enterally infused thyroid hormones. *Endocrinology* **123**, 2526-2539.

EPA, U. S. (1997). Exposure Factors Handbook Vol II Food Ingestion Factors. *US Government Printing Office* **EPA 600/P-002Fb**, 14.11-14.15.

Fang, J. L., R. L. Stingley, F. A. Beland, W. Harrouk, D. L. Lumpkins and P. Howard (2010). Occurrence, efficacy, metabolism, and toxicity of triclosan. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **28**, 147-171.

Findlay, K. A., E. Kaptein, T. J. Visser and B. Burchell (2000). Characterization of the uridine diphosphate-glucuronosyltransferase-catalyzing thyroid hormone glucuronidation in man. *J Clin Endocrinol Metab* **85**, 2879-2883.

Foran, C. M., E. R. Bennett and W. H. Benson (2000). Developmental evaluation of a potential non-steroidal estrogen: triclosan. *Marine environmental research* **50**, 153-156.

Friesema, E. C., R. Docter, E. P. Moerings, B. Stieger, B. Hagenbuch, P. J. Meier, E. P. Krenning, G. Hennemann and T. J. Visser (1999). Identification of thyroid hormone transporters. *Biochemical and biophysical research communications* **254**, 497-501.

Friesema, E. C., J. Jansen, C. Milici and T. J. Visser (2005). Thyroid hormone transporters. *Vitamins and hormones* **70**, 137-167.

Galton, V. A. and B. C. Nisula (1972). The enterohepatic circulation of thyroxine. *The Journal of endocrinology* **54**, 187-193.

Gauger, K. J., Y. Kato, K. Haraguchi, H. J. Lehmler, L. W. Robertson, R. Bansal and R. T. Zoeller (2004). Polychlorinated biphenyls (PCBs) exert thyroid hormone-like effects in the fetal rat brain but do not bind to thyroid hormone receptors. *Environmental health perspectives* **112**, 516-523.

Gauger, K. J., S. Giera, D. S. Sharlin, R. Bansal, E. Iannacone and R. T. Zoeller (2007). Polychlorinated biphenyls 105 and 118 form thyroid hormone receptor agonists after cytochrome P4501A1 activation in rat pituitary GH3 cells. *Environmental health perspectives* **115**, 1623-1630.

Gee, R. H., A. Charles, N. Taylor and P. D. Darbre (2008). Oestrogenic and androgenic activity of triclosan in breast cancer cells. *J Appl Toxicol* **28**, 78-91.

Gereben, B., A. M. Zavacki, S. Ribich, B. W. Kim, S. A. Huang, W. S. Simonides, A. Zeold and A. C. Bianco (2008). Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev* **29**, 898-938.

Gittoes, N. and J. A. Franklyn (1995). Drug-Induced Thyroid Disorders. *Drug Safety* **13**, 46-55.

Glinoer, D. (1997). The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocr Rev* **18**, 404-433.

Gonzalez-Sancho, J. M., V. Garcia, F. Bonilla and A. Munoz (2003). Thyroid hormone receptors/THR genes in human cancer. *Cancer Lett* **192**, 121-132.

Gonzalez, F. J. and Y. M. Shah (2008). PPARalpha: mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology* **246**, 2-8.

Gordin, F. M., M. E. Schultz, R. A. Huber and J. A. Gill (2005). Reduction in nosocomial transmission of drug-resistant bacteria after introduction of an alcohol-based handrub. *Infect Control Hosp Epidemiol* **26**, 650-653.

Grijota-Martinez, C., E. Samarut, T. S. Scanlan, B. Morte and J. Bernal (2011). In Vivo Activity of the Thyroid Hormone Receptor {beta}- and {alpha}-Selective Agonists GC-24 and CO23 on Rat Liver, Heart, and Brain. *Endocrinology*.

Guillen, J., A. Bernabeu, S. Shapiro and J. Villalain (2004). Location and orientation of Triclosan in phospholipid model membranes. *Eur Biophys J* **33**, 448-453.

Guo, G. L., D. R. Johnson and C. D. Klaassen (2002). Postnatal expression and induction by pregnenolone-16alpha-carbonitrile of the organic anion-transporting polypeptide 2 in rat liver. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 283-288.

Haas, C. N., J. R. Marie, J. B. Rose and C. P. Gerba (2005). Assessment of benefits from use of antimicrobial hand products: reduction in risk from handling ground beef. *Int J Hyg Environ Health* **208**, 461-466.

Haddow, J. E., G. E. Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *The New England journal of medicine* **341**, 549-555.

Hanioka, N., E. Omae, T. Nishimura, H. Jinno, S. Onodera, R. Yoda and M. Ando (1996). Interaction of 2,4,4'-trichloro-2'-hydroxydiphenyl ether with microsomal cytochrome P450-dependent monooxygenases in rat liver. *Chemosphere* **33**, 265-276.

Hanioka, N., H. Jinno, T. Nishimura and M. Ando (1997). Effect of 2,4,4'-trichloro-2'-hydroxydiphenyl ether on cytochrome P450 enzymes in the rat liver. *Chemosphere* **34**, 719-730.

Heath, R. J. and C. O. Rock (2000). A triclosan-resistant bacterial enzyme. *Nature* **406**, 145-146.

Hedge, J. M., M. J. DeVito and K. M. Crofton (2009). In vivo acute exposure to polychlorinated biphenyls: effects on free and total thyroxine in rats. *Int J Toxicol* **28**, 382-391.

Hill, R. N., T. M. Crisp, P. M. Hurley, S. L. Rosenthal and D. V. Singh (1998). Risk assessment of thyroid follicular cell tumors. *Environmental health perspectives* **106**, 447-457.

Hood, A., R. Hashmi and C. D. Klaassen (1999). Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. *Toxicology and applied pharmacology* **160**, 163-170.

Hood, A. and C. D. Klaassen (2000). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**, 78-84.

Hood, A., M. L. Allen, Y. Liu, J. Liu and C. D. Klaassen (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicology and applied pharmacology* **188**, 6-13.

Houtman, C. J., A. M. Van Oostveen, A. Brouwer, M. H. Lamoree and J. Legler (2004). Identification of estrogenic compounds in fish bile using bioassay-directed fractionation. *Environmental science & technology* **38**, 6415-6423.

Hovander, L., T. Malmberg, M. Athanasiadou, I. Athanassiadis, S. Rahm, A. Bergman and E. K. Wehler (2002). Identification of hydroxylated PCB metabolites and other phenolic halogenated pollutants in human blood plasma. *Archives of environmental contamination and toxicology* **42**, 105-117.

Howdeshell, K. L. (2002). A model of the development of the brain as a construct of the thyroid system. *Environmental health perspectives* **110 Suppl 3**, 337-348.

Hurley, P. M. (1998). Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. *Environmental health perspectives* **106**, 437-445.

Ibarrola, N. and A. Rodriguez-Pena (1997). Hypothyroidism coordinately and transiently affects myelin protein gene expression in most rat brain regions during postnatal development. *Brain Res* **752**, 285-293.

Iniguez, M. A., L. De Lecea, A. Guadano-Ferraz, B. Morte, D. Gerendasy, J. G. Sutcliffe and J. Bernal (1996). Cell-specific effects of thyroid hormone on RC3/neurogranin expression in rat brain. *Endocrinology* **137**, 1032-1041.

Ishibashi, H., N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao and K. Arizono (2004). Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. *Aquatic toxicology (Amsterdam, Netherlands)* **67**, 167-179.

Jacobs, M. N., G. T. Nolan and S. R. Hood (2005). Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicology and applied pharmacology* **209**, 123-133.

Jansen, J., E. C. Friesema, C. Milici and T. J. Visser (2005). Thyroid hormone transporters in health and disease. *Thyroid* **15**, 757-768.

Jinno, H., N. Hanioka, S. Onodera, T. Nishimura and M. Ando (1997). Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel. *Xenobiotica; the fate of foreign compounds in biological systems* **27**, 681-692.

Johnson, D. R., S. S. Habeebu and C. D. Klaassen (2002a). Increase in bile flow and biliary excretion of glutathione-derived sulfhydryls in rats by drug-metabolizing enzyme inducers is mediated by multidrug resistance protein 2. *Toxicol Sci* **66**, 16-26.

Johnson, D. R. and C. D. Klaassen (2002b). Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. *Toxicol Sci* **67**, 182-189.

Johnson, E. F., C. N. Palmer, K. J. Griffin and M. H. Hsu (1996). Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *FASEB J* **10**, 1241-1248.

Johnson, E. F., M. H. Hsu, U. Savas and K. J. Griffin (2002c). Regulation of P450 4A expression by peroxisome proliferator activated receptors. *Toxicology* **181-182**, 203-206.

Jones, S. A., L. B. Moore, J. L. Shenk, G. B. Wisely, G. A. Hamilton, D. D. McKee, N. C. Tomkinson, E. L. LeCluyse, M. H. Lambert, T. M. Willson, S. A. Kliewer and J. T. Moore (2000). The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Molecular endocrinology (Baltimore, Md* **14**, 27-39.

Kanda, R., P. Griffin, H. A. James and J. Fothergill (2003). Pharmaceutical and personal care products in sewage treatment works. *J Environ Monit* **5**, 823-830.

Kapoor, R., M. van Hogerlinden, K. Wallis, H. Ghosh, K. Nordstrom, B. Vennstrom and V. A. Vaidya (2010). Unliganded thyroid hormone receptor alpha1 impairs adult hippocampal neurogenesis. *FASEB J* **24**, 4793-4805.

Kaptein, E., G. A. van Haasteren, E. Linkels, W. J. de Greef and T. J. Visser (1997). Characterization of iodothyronine sulfotransferase activity in rat liver. *Endocrinology* **138**, 5136-5143.

Kato, Y., H. Suzuki, S. Ikushiro, S. Yamada and M. Degawa (2005). Decrease in serum thyroxine level by phenobarbital in rats is not necessarily dependent on increase in hepatic UDP-glucuronosyltransferase. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1608-1612.

Klaassen, C. D. and A. L. Slitt (2005). Regulation of hepatic transporters by xenobiotic receptors. *Current drug metabolism* **6**, 309-328.

Klaunig, J. E., M. A. Babich, K. P. Baetcke, J. C. Cook, J. C. Corton, R. M. David, J. G. DeLuca, D. Y. Lai, R. H. McKee, J. M. Peters, R. A. Roberts and P. A. Fenner-Crisp (2003). PPARalpha agonist-induced rodent tumors: modes of action and human relevance. *Critical reviews in toxicology* **33**, 655-780.

Kohn, M. C. (2000). Effects of TCDD on thyroid hormone homeostasis in the rat. *Drug Chem Toxicol* **23**, 259-277.

Kohrle, J. (1996). Thyroid hormone deiodinases--a selenoenzyme family acting as gate keepers to thyroid hormone action. *Acta medica Austriaca* **23**, 17-30.

Kohrle, J. (2002). Iodothyronine deiodinases. *Methods in enzymology* **347**, 125-167.

Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber and H. T. Buxton (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environmental science & technology* **36**, 1202-1211.

Kooistra, L., S. Crawford, A. L. van Baar, E. P. Brouwers and V. J. Pop (2006). Neonatal effects of maternal hypothyroxinemia during early pregnancy. *Pediatrics* **117**, 161-167.

Kretschmer, X. C. and W. S. Baldwin (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chemico-biological interactions* **155**, 111-128.

Langdon, K. A., M. S. Warne and R. S. Kookana (2010). Aquatic hazard assessment for pharmaceuticals, personal care products, and endocrine-disrupting compounds from biosolids-amended land. *Integr Environ Assess Manag* **6**, 663-676.

Lans, M. C., C. Spiertz, A. Brouwer and J. H. Koeman (1994). Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. *Eur J Pharmacol* **270**, 129-136.

Lavado-Autric, R., E. Auso, J. V. Garcia-Velasco, C. Arufe Mdel, F. Escobar del Rey, P. Berbel and G. Morreale de Escobar (2003). Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. *The Journal of clinical investigation* **111**, 1073-1082.

Lecureux, L., M. Z. Dieter, D. M. Nelson, L. Watson, H. Wong, B. Gemzik, C. D. Klaassen and L. D. Lehman-McKeeman (2009). Hepatobiliary disposition of thyroid hormone in Mrp2-deficient TR- rats: reduced biliary excretion of thyroxine glucuronide does not prevent xenobiotic-induced hypothyroidism. *Toxicol Sci* **108**, 482-491.

Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice and J. B. Rafferty (1999). Molecular basis of triclosan activity. *Nature* **398**, 383-384.

Li, Y., Z. Shan, W. Teng, X. Yu, C. Fan, X. Teng, R. Guo, H. Wang, J. Li, Y. Chen, W. Wang, M. Chawinga, L. Zhang, L. Yang, Y. Zhao and T. Hua (2010). Abnormalities of maternal thyroid function during pregnancy affect neuropsychological development of their children at 25-30 months. *Clinical endocrinology* **72**, 825-829.

Lin, Y. J. (2000). Buccal absorption of triclosan following topical mouthrinse application. *American journal of dentistry* **13**, 215-217.

Liu, J., Y. Liu, R. A. Barter and C. D. Klaassen (1995). Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *The Journal of pharmacology and experimental therapeutics* **273**, 977-985.

Lopez-Avila, V., Hites R.A. (1980). Organic compounds in industrial wastewater: their transport into sediments. *Environmental Science and Technology* **14**.

Loraine, G. A. and M. E. Pettigrove (2006). Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. *Environmental science & technology* **40**, 687-695.

Luo, G., M. Cunningham, S. Kim, T. Burn, J. Lin, M. Sinz, G. Hamilton, C. Rizzo, S. Jolley, D. Gilbert, A. Downey, D. Mudra, R. Graham, K. Carroll, J. Xie, A. Madan, A. Parkinson, D. Christ, B. Selling, E. LeCluyse and L. S. Gan (2002). CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 795-804.

Lyman, F. L. and T. Furia (1969). Toxicology of 2, 4, 4'-trichloro-2'-hydroxy-diphenyl ether. *IMS, Industrial medicine and surgery* **38**, 64-71.

Matsumura, N., H. Ishibashi, M. Hirano, Y. Nagao, N. Watanabe, H. Shiratsuchi, T. Kai, T. Nishimura, A. Kashiwagi and K. Arizono (2005). Effects of nonylphenol and triclosan on production of plasma vitellogenin and testosterone in male South African clawed frogs (Xenopus laevis). *Biological & pharmaceutical bulletin* **28**, 1748-1751.

McAvoy, D. C., B. Schatowitz, M. Jacob, A. Hauk and W. S. Eckhoff (2002). Measurement of triclosan in wastewater treatment systems. *Environmental toxicology and chemistry / SETAC* **21**, 1323-1329.

McMurry, L. M., M. Oethinger and S. B. Levy (1998a). Triclosan targets lipid synthesis. *Nature* **394**, 531-532.

McMurry, L. M., M. Oethinger and S. B. Levy (1998b). Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of Escherichia coli. *FEMS microbiology letters* **166**, 305-309.

Michels, A. W. and G. S. Eisenbarth (2010). Immunologic endocrine disorders. *J Allergy Clin Immunol* **125**, S226-237.

Miller, M. D., K. M. Crofton, D. C. Rice and R. T. Zoeller (2009). Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. *Environmental health perspectives* **117**, 1033-1041.

Moriyama, K., T. Tagami, T. Akamizu, T. Usui, M. Saijo, N. Kanamoto, Y. Hataya, A. Shimatsu, H. Kuzuya and K. Nakao (2002). Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J Clin Endocrinol Metab* **87**, 5185-5190.

Morreale de Escobar, G., M. J. Obregon and F. Escobar del Rey (2000). Is neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia? *J Clin Endocrinol Metab* **85**, 3975-3987.

Morreale de Escobar, G., M. J. Obregon and F. Escobar del Rey (2004). Role of thyroid hormone during early brain development. *European journal of endocrinology / European Federation of Endocrine Societies* **151 Suppl 3**, U25-37.

Morse, D. C., D. Groen, M. Veerman, C. J. van Amerongen, H. B. Koeter, A. E. Smits van Prooije, T. J. Visser, J. H. Koeman and A. Brouwer (1993). Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. *Toxicology and applied pharmacology* **122**, 27-33.

Moss, T., D. Howes and F. M. Williams (2000). Percutaneous penetration and dermal metabolism of triclosan (2,4, 4'-trichloro-2'-hydroxydiphenyl ether). *Food Chem Toxicol* **38**, 361-370.

Mottaleb, M. A., S. Usenko, J. G. O'Donnell, A. J. Ramirez, B. W. Brooks and C. K. Chambliss (2009). Gas chromatography-mass spectrometry screening methods for select UV filters, synthetic musks, alkylphenols, an antimicrobial agent, and an insect repellent in fish. *Journal of chromatography* **1216**, 815-823.

Myant, N. B. (1956). Enterohepatic circulation of thyroxine in humans. *Clin Sci (Lond)* **15**, 551-555.

Nakada, N., T. Tanishima, H. Shinohara, K. Kiri and H. Takada (2006). Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. *Water research* **40**, 3297-3303.

Nishi, I., T. Kawakami and S. Onodera (2008). Monitoring of triclosan in the surface water of the Tone Canal, Japan. *Bulletin of environmental contamination and toxicology* **80**, 163-166.

Omiecinski, C. J., V. H. J.P., G. H. Perdew and J. M. Peters (2011). Xenobiotic Metabolism, Disposition, and Regulation by Receptors: From Biochemical Phenomenon to Predictors of Major Toxicities. *Toxicol Sci* **120**, S49-75.

Orvos, D. R., D. J. Versteeg, J. Inauen, M. Capdevielle, A. Rothenstein and V. Cunningham (2002). Aquatic toxicity of triclosan. *Environmental toxicology and chemistry / SETAC* **21**, 1338-1349.

Oscarson, M., U. M. Zanger, O. F. Rifki, K. Klein, M. Eichelbaum and U. A. Meyer (2006). Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. *Clin Pharmacol Ther* **80**, 440-456.

Park, H. Y., J. S. Park, E. Sovcikova, A. Kocan, L. Linderholm, A. Bergman, T. Trnovec and I. Hertz-Picciotto (2009). Exposure to hydroxylated polychlorinated biphenyls (OH-PCBs) in the prenatal period and subsequent neurodevelopment in eastern Slovakia. *Environmental health perspectives* **117**, 1600-1606.

Paxeus, N. (1996). Organic pollutants in effluents of large wastewater treatment plants in Sweden. *Water research* **30**, 1115-1122.

Perencevich, E. N., M. T. Wong and A. D. Harris (2001). National and regional assessment of the antibacterial soap market: a step toward determining the impact of prevalent antibacterial soaps. *American journal of infection control* **29**, 281-283.

Perucca, E., A. Hedges, K. A. Makki, M. Ruprah, J. F. Wilson and A. Richens (1984). A comparative study of the relative enzyme inducing properties of anticonvulsant drugs in epileptic patients. *Br J Clin Pharmacol* **18**, 401-410.

Pop, V. J., J. L. Kuijpens, A. L. van Baar, G. Verkerk, M. M. van Son, J. J. de Vijlder, T. Vulsma, W. M. Wiersinga, H. A. Drexhage and H. L. Vader (1999). Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clinical endocrinology* **50**, 149-155.

Pop, V. J., E. P. Brouwers, H. L. Vader, T. Vulsma, A. L. van Baar and J. J. de Vijlder (2003). Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clinical endocrinology* **59**, 282-288.

Porterfield, S. P. and C. E. Hendrich (1993). The role of thyroid hormones in prenatal and neonatal neurological development--current perspectives. *Endocr Rev* **14**, 94-106.

Ramirez, A. J., R. A. Brain, S. Usenko, M. A. Mottaleb, J. G. O'Donnell, L. L. Stahl, J. B. Wathen, B. D. Snyder, J. L. Pitt, P. Perez-Hurtado, L. L. Dobbins, B. W. Brooks and C. K.

Chambliss (2009). Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. *Environmental toxicology and chemistry / SETAC* **28**, 2587-2597.

Reiss, R., N. Mackay, C. Habig and J. Griffin (2002). An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. *Environmental toxicology and chemistry / SETAC* **21**, 2483-2492.

Rice, D. and S. Barone, Jr. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental health perspectives* **108 Suppl 3**, 511-533.

Richardson, T. A. and C. D. Klaassen (2010). Disruption of thyroid hormone homeostasis in Ugt1a-deficient Gunn rats by microsomal enzyme inducers is not due to enhanced thyroxine glucuronidation. *Toxicology and applied pharmacology* **248**, 38-44.

Ritter, J. K., F. K. Kessler, M. T. Thompson, A. D. Grove, D. J. Auyeung and R. A. Fisher (1999). Expression and inducibility of the human bilirubin UDP-glucuronosyltransferase UGT1A1 in liver and cultured primary hepatocytes: evidence for both genetic and environmental influences. *Hepatology* **30**, 476-484.

Rodricks, J. V., J. A. Swenberg, J. F. Borzelleca, R. R. Maronpot and A. M. Shipp (2010). Triclosan: a critical review of the experimental data and development of margins of safety for consumer products. *Critical reviews in toxicology* **40**, 422-484.

Royland, J. E., J. S. Parker and M. E. Gilbert (2008). A genomic analysis of subclinical hypothyroidism in hippocampus and neocortex of the developing rat brain. *Journal of neuroendocrinology* **20**, 1319-1338.

Saleh, S., R. N. Haddadin, S. Baillie and P. J. Collier (2011). Triclosan - an update. *Lett Appl Microbiol* **52**, 87-95.

Sandborgh-Englund, G., M. Adolfsson-Erici, G. Odham and J. Ekstrand (2006). Pharmacokinetics of triclosan following oral ingestion in humans. *Journal of toxicology and environmental health* **69**, 1861-1873.

Savu, L., R. Vranckx, M. Maya and E. A. Nunez (1987). A thyroxine binding globulin (TBG)-like protein in the sera of developing and adult rats. *Biochemical and biophysical research communications* **148**, 1165-1173.

Schmutzler, C., A. Bacinski, I. Gotthardt, K. Huhne, P. Ambrugger, H. Klammer, C. Schlecht, C. Hoang-Vu, A. Gruters, W. Wuttke, H. Jarry and J. Kohrle (2007). The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent in vitro inhibitor of human recombinant thyroid peroxidase. *Endocrinology* **148**, 2835-2844.

Schussler, G. C. (2000). The thyroxine-binding proteins. *Thyroid* **10**, 141-149.

Schuur, A. G., F. M. Boekhorst, A. Brouwer and T. J. Visser (1997). Extrathyroidal effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on thyroid hormone turnover in male Sprague-Dawley rats. *Endocrinology* **138**, 3727-3734.

See, N. A. (1996). Review and Evaluation of Pharmacology and Toxicology Data Division of Dermatologic and Dental Products *Food and Drug Administration* **HFD-540**.

Sharlin, D. S., D. Tighe, M. E. Gilbert and R. T. Zoeller (2008). The balance between oligodendrocyte and astrocyte production in major white matter tracts is linearly related to serum total thyroxine. *Endocrinology* **149**, 2527-2536.

Sickbert-Bennett, E. E., D. J. Weber, M. F. Gergen-Teague, M. D. Sobsey, G. P. Samsa and W. A. Rutala (2005). Comparative efficacy of hand hygiene agents in the reduction of bacteria and viruses. *American journal of infection control* **33**, 67-77.

Siddiqui, W. H. and H. S. Buttar (1979). Pharmacokinetics of triclosan in rat after intravenous and intravaginal administration. *Journal of environmental pathology and toxicology* **2**, 861-871.

Simko, J. and J. Horacek (2007). Carbamazepine and risk of hypothyroidism: a prospective study. *Acta Neurol Scand* **116**, 317-321.

Singer, H., S. Muller, C. Tixier and L. Pillonel (2002). Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environmental science & technology* **36**, 4998-5004.

Smirlis, D., R. Muangmoonchai, M. Edwards, I. R. Phillips and E. A. Shephard (2001). Orphan receptor promiscuity in the induction of cytochromes p450 by xenobiotics. *J Biol Chem* **276**, 12822-12826.

Stoker, T. E., E. K. Gibson and L. M. Zorrilla (2010). Triclosan exposure modulates estrogen-dependent responses in the female wistar rat. *Toxicol Sci* **117**, 45-53.

Tatarazako, N., H. Ishibashi, K. Teshima, K. Kishi and K. Arizono (2004). Effects of triclosan on various aquatic organisms. *Environ Sci* **11**, 133-140.

Timsit, Y. E. and M. Negishi (2007). CAR and PXR: the xenobiotic-sensing receptors. *Steroids* **72**, 231-246.

Tulp, M. T., G. Sundstrom, L. B. Martron and O. Hutzinger (1979). Metabolism of chlorodiphenyl ethers and Irgasan DP 300. *Xenobiotica; the fate of foreign compounds in biological systems* **9**, 65-77.

USEPA (2008). Reregistration Eligibility Decision for Triclosan (P. Office of Prevention, and Toxic Substances, Ed.^ Eds.), pp. 1-47.

van den Berg, K. J. (1990). Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin and thyroid binding globulin. *Chemico-biological interactions* **76**, 63-75.

Vansell, N. R. and C. D. Klaassen (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicology and applied pharmacology* **176**, 187-194.

Vansell, N. R. and C. D. Klaassen (2002). Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 240-246.

Veldhoen, N., R. C. Skirrow, H. Osachoff, H. Wigmore, D. J. Clapson, M. P. Gunderson, G. Van Aggelen and C. C. Helbing (2006). The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquatic toxicology (Amsterdam, Netherlands)* **80**, 217-227.

Visser, T. J., E. van Overmeeren, D. Fekkes, R. Docter and G. Hennemann (1979). Inhibition of iodothyronine 5'-deiodinase by thioureylenes; structure--activity relationship. *FEBS Lett* **103**, 314-318.

Visser, T. J., M. Rutgers, W. W. de Herder, S. J. Rooda and M. P. Hazenberg (1988). Hepatic metabolism, biliary clearance and enterohepatic circulation of thyroid hormone. *Acta medica Austriaca* **15 Suppl 1**, 37-39.

Visser, T. J., J. C. van Buuren, M. Rutgers, S. J. Eelkman Rooda and W. W. de Herder (1990). The role of sulfation in thyroid hormone metabolism. *Trends Endocrinol Metab* **1**, 211-218.

Visser, T. J., E. Kaptein, H. van Toor, J. A. van Raaij, K. J. van den Berg, C. T. Joe, J. G. van Engelen and A. Brouwer (1993). Glucuronidation of thyroid hormone in rat liver: effects of in vivo treatment with microsomal enzyme inducers and in vitro assay conditions. *Endocrinology* **133**, 2177-2186.

Visser, T. J. (1996). Pathways of thyroid hormone metabolism. *Acta medica Austriaca* **23**, 10-16.

Vranckx, R., M. Rouaze, L. Savu, E. A. Nunez, C. Beaumont and I. L. Flink (1990). The hepatic biosynthesis of rat thyroxine binding globulin (TBG): demonstration, ontogenesis, and up-regulation in experimental hypothyroidism. *Biochemical and biophysical research communications* **167**, 317-322.

Vranckx, R., M. Rouaze-Romet, L. Savu, P. Mechighel, M. Maya and E. A. Nunez (1994). Regulation of rat thyroxine-binding globulin and transthyretin: studies in thyroidectomized

and hypophysectomized rats given tri-iodothyronine or/and growth hormone. *The Journal of endocrinology* **142**, 77-84.

Wallis, K., S. Dudazy, M. van Hogerlinden, K. Nordstrom, J. Mittag and B. Vennstrom (2010). The thyroid hormone receptor alpha1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. *Molecular endocrinology (Baltimore, Md* **24**, 1904-1916.

Waltman, E. L., B. J. Venables and W. T. Waller (2006). Triclosan in a north Texas wastewater treatment plant and the influent and effluent of an experimental constructed wetland. *Environmental toxicology and chemistry / SETAC* **25**, 367-372.

Wang, L. Q. and M. O. James (2006). Inhibition of sulfotransferases by xenobiotics. *Current drug metabolism* **7**, 83-104.

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

Williams, G. R. (2008). Neurodevelopmental and neurophysiological actions of thyroid hormone. *Journal of neuroendocrinology* **20**, 784-794.

Wolff, J. (1998). Perchlorate and the thyroid gland. *Pharmacol Rev* **50**, 89-105.

Wolff, M. S., S. L. Teitelbaum, G. Windham, S. M. Pinney, J. A. Britton, C. Chelimo, J. Godbold, F. Biro, L. H. Kushi, C. M. Pfeiffer and A. M. Calafat (2007). Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. *Environmental health perspectives* **115**, 116-121.

Wong, H., L. D. Lehman-McKeeman, M. F. Grubb, S. J. Grossman, V. M. Bhaskaran, E. G. Solon, H. S. Shen, R. J. Gerson, B. D. Car, B. Zhao and B. Gemzik (2005). Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. *Toxicol Sci* **84**, 232-242.

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

Yamanaka, H., M. Nakajima, M. Katoh and T. Yokoi (2007). Glucuronidation of thyroxine in human liver, jejunum, and kidney microsomes. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 1642-1648.

Yazdankhah, S. P., A. A. Scheie, E. A. Hoiby, B. T. Lunestad, E. Heir, T. O. Fotland, K. Naterstad and H. Kruse (2006). Triclosan and antimicrobial resistance in bacteria: an overview. *Microbial drug resistance (Larchmont, N.Y* **12**, 83-90.

Yen, P. M. (2001). Physiological and molecular basis of thyroid hormone action. *Physiol Rev* **81**, 1097-1142.

Zamoner, A., L. Heimfarth, S. Oliveira Loureiro, C. Royer, F. R. Mena Barreto Silva and R. Pessoa-Pureur (2008). Nongenomic actions of thyroxine modulate intermediate filament phosphorylation in cerebral cortex of rats. *Neuroscience* **156**, 640-652.

Zhou, T., D. G. Ross, M. J. DeVito and K. M. Crofton (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zhou, T., M. M. Taylor, M. J. DeVito and K. M. Crofton (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci* **66**, 105-116.

Zoeller, R. T. and K. M. Crofton (2000). Thyroid hormone action in fetal brain development and potential for disruption by environmental chemicals. *Neurotoxicology* **21**, 935-945.

Zoeller, R. T. and D. C. Rice (2004). Critical effect of perchlorate on neonates is iodide uptake inhibition. *Regul Toxicol Pharmacol* **40**, 376-377; author reply 378-379.

Zoeller, R. T., R. Bansal and C. Parris (2005). Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. *Endocrinology* **146**, 607-612.

Zoeller, R. T., S. W. Tan and R. W. Tyl (2007a). General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Critical reviews in toxicology* **37**, 11-53.

Zoeller, R. T., R. W. Tyl and S. W. Tan (2007b). Current and potential rodent screens and tests for thyroid toxicants. *Critical reviews in toxicology* **37**, 55-95.

Zorrilla, L. M., E. K. Gibson, S. C. Jeffay, K. M. Crofton, W. R. Setzer, R. L. Cooper and T. E. Stoker (2009). The effects of triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol Sci* **107**, 56-64.

Short-term Exposure to Triclosan Decreases Thyroxine In Vivo via Upregulation of Hepatic Catabolism in Young Long-Evans Rats

Chapter 2

Katie B. Paul[1], Joan M. Hedge[2], Michael J. DeVito[2], Kevin M. Crofton[1,2]

[1]Curriculum in Toxicology, UNC, Chapel Hill, NC; [2]Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, ORD, U.S. EPA, RTP, NC.

[‡]Correspondence: Kevin M. Crofton, Ph.D., Neurotoxicology Division, MD-B105-04, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; phone: 919-541-2672; email: crofton.kevin@epa.gov

1. Abstract

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a chlorinated phenolic antibacterial compound found in consumer products. *In vitro* human pregnane X receptor (PXR) activation, hepatic Phase I enzyme induction, and decreased *in vivo* total thyroxine (T4) suggest adverse effects on thyroid hormone homeostasis. Current research tested the hypothesis that triclosan decreases circulating T4 via upregulation of hepatic catabolism and transport. Weanling female Long-Evans rats received triclosan (0-1000 mg/kg/day) by gavage for four days. Whole blood and liver were collected 24 hrs later. Total serum T4, triiodothyronine (T3), and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay. Hepatic microsomal assays measured ethoxyresorufin-O-deethylase (EROD), pentoxyresorufin-O-deethylase (PROD), and uridine diphosphate glucuronyltransferase (UGT) enzyme activities. The mRNA expression of cytochrome P450s *1a1*, *2b1/2*, and *3a1/23*, UGTs *1a1*, *1a6*, and *2b5*, sulfotransferases *1c1* and *1b1*, and hepatic transporters *Oatp1a1*, *Oatp1a4*, *Mrp2*, and *Mdr1b* was measured by quantitative RT-PCR. Total T4 decreased dose-responsively, down to 43% of control at 1000 mg/kg/day. Total T3 was decreased to 89% and 75% of control at 300 and 1000 mg/kg/day. TSH did not change. Triclosan dose-dependently increased PROD activity up to 900 percent of control at 1000 mg/kg/day. T4-glucuronidation increased nearly 2-fold at 1000 mg/kg/day. *Cyp2b1/2* and *Cyp3a1/23* mRNA expression levels were induced 2-fold and 4-fold at 300 mg/kg/day. *Ugt1a1* and *Sult1c1* mRNA expression increased 2.2-fold and 2.6-fold at 300 mg/kg/day. Transporter mRNA expression levels were unchanged. These data denote important key events in the mode-of-action for triclosan-induced hypothyroxinemia in rats, and suggest that this effect may be partially due to upregulation of hepatic catabolism, but not due to mRNA expression changes in the tested hepatic transporters.

2. Introduction

Triclosan is a chlorinated phenolic bacteriocide used as an active ingredient in personal care products, including soaps, toothpastes, toys, and clothing (Bhargava & Leonard 1996, Dayan 2007, McClanahan & Bartizek 2002). Due to broad use, environmental fate and transport, triclosan is an environmental contaminant (Kolpin et al. 2002, Paxeus 1996). Triclosan contamination of effluents is one of several potential sources for human exposure, along with personal care product exposure (Sandborgh-Englund et al. 2006, Allmyr et al. 2006).

Triclosan is readily absorbed from the gastrointestinal tract and oral mucosa (Sandborgh-Englund et al. 2006). Triclosan has been detected in human breast milk, at levels ranging from 0 – 2100 µg/kg-lipid (Adolfsson-Erici et al. 2002, Allmyr et al. 2006, Dayan 2007), in serum (Allmyr et al. 2006, Hovander et al. 2002), and as triclosan metabolites in urine (Calafat et al. 2008, Wolff et al. 2007). These data demonstrate exposure in humans, and underscore the importance of using models to assess any potential effects resulting from human triclosan exposure.

Laboratory studies have documented that triclosan disrupts thyroid systems in rats and frogs. (Crofton et al. 2007, Zorrilla et al. 2009, Veldhoen et al. 2006). *In vitro* evidence also suggests that triclosan may be an antagonist (Gee et al. 2008, Ahn et al. 2008) or weak agonist (Houtman et al. 2004) of the estrogen receptor, and/or a weak antagonist of the androgen receptor (Chen et al. 2007).

Adverse effects of triclosan on thyroid hormone (TH) homeostasis have been reported in rats and frogs. Short-term oral triclosan exposure resulted in hypothyroxinemia in weanling rats (Crofton et al. 2007). Stoker and colleagues reported that triclosan decreased T4 without significantly affecting thyroid stimulating hormone (TSH) following a 31-day oral triclosan

exposure (Zorrilla et al. 2009). Veldhoen *et al.* (2006) reported increased thyroid receptor β (TRβ) mRNA expression in *Xenopus laevis* XTC-2 cells when co-exposed to T3, and accelerated hind limb development *in vivo* in *Rana catesbeina* (Veldhoen et al. 2006). The mode-of-action for thyroid disruption by triclosan is currently undefined.

Thyroid disruption may occur via several mechanisms, including disruption of TH synthesis via inhibition of thyroperoxidase or iodide uptake; binding to TH transport proteins; xenobiotic interactions with the TH receptor or thyroid stimulating hormone receptor; upregulation of iodothyronine deiodinases; and increases in TH clearance (Boas et al. 2006, Crofton 2008, Capen 1994). Evidence suggests that triclosan may upregulate hepatic catabolism of THs, a known key event in a mode-of-action leading to decreased THs (Crofton 2008, Crofton & Zoeller 2005, Hill et al. 1998, McClain et al. 1989). The initiating event in this mode-of-action is the activation of hepatic nuclear receptors (Crofton & Zoeller 2005, Hill et al. 1998, McClain 1989, Barter & Klaassen 1994, Hood & Klaassen 2000). Nuclear receptors PXR and CAR regulate hepatic catabolic and transport activity (Kretschmer & Baldwin 2005), suggesting that potential agonism or antagonism of CAR/PXR results in downstream metabolic changes that affect serum TH levels. In PXR reporter assays, triclosan moderately activated human PXR relative to the prototypical inducer, rifampicin (Jacobs et al. 2005). Further, triclosan increased Cyp2b1/2 and Cyp3a1 protein and enzymatic activity markers of CYP activity including pentoxyresorufin-O-dependentylase (PROD), benzyloxyresorufin-O-debentylase (BROD), and ethoxyresorufin-O-deethylase (EROD) in hepatocytes *in vitro* and in hepatic microsomes *ex vivo* (Jinno et al. 1997, Zorrilla et al. 2009, Hanioka et al. 1996). Triclosan inhibited diiodothyronine (T2) sulfotransferases in rat and human hepatocytes *in vitro*, suggesting interference with thyroid hormone sulfation (Wang & James 2006, Wang et al. 2004). Available evidence suggests

activation of hepatic nuclear receptors by triclosan, with subsequent upregulation of hepatic metabolism, which contributes to the maintenance of thyroid homeostasis (Capen 1994, Hill et al. 1998, DeVito et al. 1999).

The current work tested the hypothesis that triclosan decreased serum T4 *in vivo* by upregulation of hepatic catabolism and altered expression of cellular transport proteins. A four-day exposure weanling rat model was employed, as this protocol has been used to characterize thyroid disrupting chemicals (Zhou et al. 2001, Craft et al. 2002, Crofton 2004), and demonstrate triclosan-induced hypothyroxinemia (Crofton et al. 2007) and hepatic enzyme induction (Hanioka et al. 1996). Serum total T4, T3, and TSH were measured. Cytochrome P450 markers of Phase I metabolism were analyzed enzymatically and as mRNA expression levels by quantitative RT-PCR. UGT activity and the expression of UGT and SULT isoforms were also measured as indicators of Phase II catabolic pathways. Finally, mRNA expression levels of several hepatic transporters were measured to determine the contribution of hepatic transport to the hypothesized mode-of-action for triclosan disruption of T4.

3. Methods

Animals. Long-Evans female rats (n=120) at 21-23 days of age were obtained from Charles River Laboratories Inc. (Raleigh, NC), and were allowed 2-4 days of acclimation in an American Association for Accreditation of Laboratory Animal Care (AALAC)-approved animal facility prior to treatment. Animals were housed two per plastic hanging cage (45 cm x 24 cm x 20 cm), with heat sterilized pine shavings bedding (Northeastern Products Corp., Warrenton, NC). Colony rooms were maintained at 21 ±2°C with 50 ±10 % humidity on a photo-period of 12L:12D (0600-1800hr). Food (Purina Rodent Chow #5001,) and water were provided *ad libitum*. Tap water (Durham, NC water) was filtered through sand, then

activated charcoal, and finally re-chlorinated to 4-5 ppm Cl^- before use in the animal facility. All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the US EPA.

The current work included hepatic tissue and serum samples from a previous publication (Crofton et al. 2007) that reported only serum T4, body weight, and liver weight values. The two experimental blocks from the previous study (Crofton et al. 2007) were combined with the additional block performed in the current study for T4 and hepatic enzyme assays; this yielded total sample sizes of n=24, 8, 24 , 24, 24, 16 for the vehicle control, 10, 30, 100, 300, and 1000 mg/kg/day, respectively. Only samples for the second block (n=8/treatment group) were used for mRNA analyses; therefore, no tissue from the 1000 mg/kg/day group was used. Only samples for the third block were used for T3 and TSH analyses (n=8/treatment group), so no tissue from the 10 mg/kg/day group was used.

Chemicals and treatment. Triclosan (5-Chloro-2-(2,4-dichloro-phenoxy)phenol, CAS#3380-34-5, LOT#06415CD) was obtained from Aldrich Chemical Company (St Louis, MO, Cat#524190-10G). Mass spectrometry, employed to analyze the triclosan sample, revealed: 98.2% triclosan, 0.05% iso-triclosan, 0.12 % 2,8,-dichlorodibenzodioxin, and 0.1% 2,4,8-trichlorodibenzodioxin. The dosing solutions (0, 10, 30, 100, 300, and 1000 mg/kg/day) were prepared in corn oil (Sigma, Lot#117K0127) and sonicated for 30 min at room temperature. The 1000 mg/kg/day dose precipitated within 24 hrs and was therefore sonicated daily before use. Dosing volume was 1.0 ml corn oil/kg body weight. Rats (27-29 days of age) were exposed via gavage for four consecutive days. Rats were randomly assigned to treatment groups to balance body weights at the start of dosing. Body weights were recorded and dosing volumes adjusted daily by weight. Approximately 24 hrs after the

final treatment rats were moved into a holding room, weighed, and acclimated for a minimum of 30 min. Trunk blood and liver were collected between 0900 and 1100 hrs in an adjacent room with a separate air supply. Blood was collected into serum separator tubes (Beckton Dickinson, 36-6154). Serum was obtained after clotting whole blood for 30 min on ice, followed by centrifugation at 1278x g at 4°C for 30 min. Livers were weighed and then quick frozen in liquid nitrogen. Serum and liver samples were stored at -80°C until analysis.

Thyroid hormone assays. Serum total T4 and serum total T3 were measured in duplicate by standard solid-phase Coat-A-Count radioimmunoassay (RIA) kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Serum TSH concentrations were analyzed in duplicates with a double antibody RIA method (Greenwood et al. 1963) with some modification (Zorrilla et al. 2009). The TSH radioimmunoassays were performed using materials supplied by the National Hormone and Pituitary Agency: iodination preparation (I-9-TSH); reference preparation (RP-3); and antisera (S-6-TSH). Iodination material was radiolabeled with ^{125}I (Perkin Elmer, Shelton, CT). Assay variation was assessed using the multivalent control module (Siemens Medical Solutions Diagnostics, Los Angeles, CA; Lot021) to measure low, medium, and high total T4, T3, and TSH values before and after measuring the experimental samples. Intra- and inter-assay coefficients of variance for all assays were below 13%. Total serum T4 and TSH were calculated as ng T4/ml serum, and total serum T3 was calculated as ng T3/dL serum.

Microsome preparation and EROD and PROD assays. Liver microsomal fractions, prepared as described previously (DeVito et al. 1993), were standardized using total protein (Bio-Rad, Richmond, CA). Hepatic microsomal EROD and PROD activities were assayed using a fluorometric microplate reader (Spectramax Gemini XPS, Molecular Devices/MDS Analytical Technologies,Toronto, Canada). Each well in polystyrene 96-well plates (Nunc

ThermoFisherScientific, Rochester, NY) contained a total of 235 ul, including 50 ul of substrate (1.5 nM ethoxyresorufin or pentoxyresorufin), 50 µL of diluted microsomes, and 110 µL of 0.05 mM Tris buffer (pH 8.0). Microsomes were diluted 1:10 for samples and 1:100 for the positive control in order to maintain data points within the linear region of the standard curve. An aliquot (25 µL) of NADPH was added to initiate the reaction. The fluorescence signal was measured every 33 seconds for five min at 37ºC after reaction initiation. The rate of resorufin formation was estimated by calculating Vmax/min, using a resorufin standard curve to extrapolate resorufin concentrations in the reaction. A similar method was used to measure hepatic microsomal PROD activity, using pentoxyresorufin as a substrate. Both EROD and PROD values were calculated as picomoles (pmol) resorufin per milligram protein per minute. A positive control, comprised of pooled microsomes from rats acutely exposed to 10 µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 300 mg/kg Aroclor, was used to facilitate inter-assay comparison.

UGT activity assay. UGT activity for T4 was measured by the method of (Beetstra et al. 1991) as modified by (Zhou et al. 2001). Detergent such as Brijj 56 was not included due to the potential for increased basal T4 glucuronidation (Craft et al. 2002). A positive control microsome pool, comprised of microsomes from rats that received a single 10 µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin dose or a single 300 mg/kg Aroclor 1254 dose, was used during each trial to enable inter-assay comparison.

mRNA preparation and analysis. Approximately 0.2 g of frozen tissue was homogenized in 2 mL of TRI Reagent (Molecular Research Center, Cincinnati, OH; Cat. No. TR 118). Total RNA was extracted per the protocol provided by the Molecular Research Center (Chomczynski & Sacchi 1987). RNA extraction was processed in duplicate. Protein, nucleic acid content, and approximate RNA concentrations were estimated with a DU800 Beckman-

Coulter Spectrophotometer via measurement of absorbance at 260 nm and 280 nm for each sample. Analysis of RNA integrity was performed using an Agilent RNA 6000 Nano Kit and Bioanalyzer, according to the instructions provided (Part Number: G2938-90033). Samples used in qRT-PCR experiments were selected from duplicates based on optimizing the 260/280 ratio to 2.0 (range =: 2.00 – 2.14). The average 28s/18s ratio for samples used was 2.2 (range = 1.9-3), and the average assigned integrity rating (RIN) was a 9.4 out of 10 (range = 8.2-10).

Gene expression assays were performed using Taqman ® One-step RT-PCR Master Mix Reagent kits (Applied Biosystems, Foster City, CA; Cat.#: 4309169), which include AmpliTaq Gold ® DNA Polymerase, dNTPs including dUTP, passive reference, and optimized buffer components for both the reverse transcriptase (RT) and DNA polymerase reactions. Also included is the RT enzyme mix, containing the MultiScribe^{TM} Reverse Transcriptase and RNase inhibitor. Taqman Gene Expression Assays (Applied Biosystems) were used to test the mRNA expression of specific genes involved in hepatic catabolism and transport (Table 1). Each 20 µL reaction contained 900 nM of the gene-specific primer and 125 ng of RNA sample, except for *OATP1a4* and *MDR1b*, which contained 400 ng of RNA per reaction. The 96-well plates were maintained at 4ºC during pipetting using a thermocycler block. Samples were analyzed in triplicate using the experimental probe and in triplicate using the endogenous control gene on each plate. Samples positions distributed across the plate based on a semi-random design, where each plate contained the same number of samples from a dose group, but the samples were randomly selected and assigned to well positions within the plate. Following sample loading, plates were centrifuged at 1400 rpm, 24ºC, for 3 min before being placed in a Stratagene MxPro3005P QPCR Detection System (Stratagene, Agilent Technologies, Cedar Creek, TX). The thermal profile was as follows:

reverse transcriptase reaction at 48ºC for 45 min, an initial 10 min denaturation period at 95ºC, followed by 60 PCR cycles of 15 sec denaturation at 94ºC, and 1 min annealing and extension at 60ºC.

The amplification efficiencies for all of the genes used in qRT-PCR experiments were confirmed using pooled, untreated control RNA extracted from female rat liver. Amplification efficiency was calculated according to the equation, Efficiency = $(10^{(-1/m)} -1) \times 100$, where m = the slope of the plot of Threshold C_T (dRn) vs. Log[RNA concentration] (Table 2.1) (Applied Biosystems Publication 127AP05-03).

qRT-PCR data sets were analyzed using a relative quantification method ($2^{-\Delta\Delta C_T}$) to describe the change in expression of the target experimental gene relative to an endogenous reference gene (Livak & Schmittgen 2001). Choice of an endogenous reference gene was based onconstant gene expression across all of the dosing groups (Dunn & Klaassen 1998), and tissue-specific amplification efficiency (Livak & Schmittgen 2001). The endogenous reference gene, Rps18, was selected after determining the amplification efficiencies of multiple candidate genes (Pohjanvirta et al. 2006), including GAPDH, β_2-microglobulin, Rps18, and Rpl13a. Choice of genes was contingent upon twocriteria: involvement of the isoform in TH metabolism and transport in rats, as well as regulation of the isoform by CAR and/or PXR

Data analysis. All three study blocks with a combined n=120 were analyzed using a two-way analysis of variance (SAS 9.1, SAS Institute, Cary, NC), with dose and block as independent variables, and T4, EROD activity, PROD activity, UGT activity as dependent variables. Significant treatment effects were followed by mean contrast testing using Duncan's New Multiple Range Test ($p<0.05$). T4, UGT, EROD, and PROD data were analyzed as mean percent of control in order to normalize the data across the three study

blocks. qRT-PCR data (Block 2 only) were analyzed using a two-way analysis of variance, with dose and plate number as independent variables, and $2^{-\Delta\Delta C}{}_T$ as the dependent variable. T3 and TSH data (Block 3 only) were analyzed using one-way analysis of variance, with dose as the independent variable and hormone as the dependent variable (SAS 9.1, SAS Institute, Cary, NC).

Benchmark dose (BMD) and lower-bound confidence limit (BMDL) estimates were determined using USEPA Benchmark Dose Software (BMDS Version 2.0.0.33) as previously described (Crofton et al. 2007, Zhou et al. 2002, Zhou et al. 2001). BMD analyses were performed to further define dose-dependent effects that are not discernible using only information on the statistically significant effects found using ANOVA. Use of BMDs enables estimation of the dose at which a specified effect, or benchmark response (BMR) may occur. The BMR was set at a 20% decrease for T4 and T3 data (Crofton et al. 2007, Zorrilla et al. 2009), 50% for increases in hepatic PROD activity, and 20% for changes in mRNA expression and UGT activity data (Schlecht et al. 2006). These BMRs were selected based on previous use in the literature, a need to balance the statistical and perceived biological significance of a change in the measured endpoint, and known mechanistic relationships (e.g., UGT and T4) (EPA 2000). The BMDs were calculated from the model fits to the data. The BMDL (lower-bound confidence limit) was calculated as the 95% lower confidence interval.

4. Results

No clinical signs of toxicity were observed in the rats following the 4 days of triclosan treatment. Effects on liver weight and liver-body weight ratio were consistent with a previously published four-day triclosan exposure model (Crofton et al. 2007). There was a main effect of dose [$F(5,105)=2.57$, $p<0.0309$] and study block [$F(2,105)=6.24$, $p<0.0027$]

on liver weight that resulted in a 12% increase liver weight in the 1000 mg/kg/day dose group. Increases in liver weight, and non-significant decreases in body weight gain of approximately 10% in the high dose group, were reflected in the main effect of dose [$F(5, 105)=18.62$, $p<0.001$] and study block [$F(5, 105)=3.50$, $p<0.0338$] on the liver-body weight ratio, which resulted in approximately a 13% increase in the liver-body weight ratio for the 1000 mg/kg/day dose group (Table 2.2).

Total serum T4 and total serum T3 both decreased in a dose-responsive manner, with decreases of 26%, 35%, and 57% relative to control at 100, 300, and 1000 mg/kg/day for T4, and decreases of 12% and 25% at 300 and 1000 mg/kg/day for T3, respectively (Figure 2.1). TSH was unchanged. For T4, there was a main effect of dose [$F(5, 105)=37.46$, $p<0.0001$], but no effect of study block ($p<0.05$), or dose-by study block interaction ($p<0.05$). Mean contrast tests revealed significant T4 decreases in the 100, 300 and 1000 mg/kg/day groups ($p<0.05$). There was also a main effect of dose on T3 concentrations [$F(4, 35)=12.80$, $p<0.0001$]; mean contrast tests demonstrated significant decreases at 300 and 1000 mg/kg/day only ($p<0.05$). The no-observed effect level (NOEL) for T4 decreases was 30 mg/kg/day, and 100 mg/kg/day for T3 decreases. Using a BMR equal to a 20% decrease in thyroid hormones, Hill model fits to the data predicted BMDs of 99.4 and 606 mg/kg/d, with 95% lower confidence limits of 65.6 and 335 mg/kg/day, for T4 and T3 respectively.

EROD activity was depressed by triclosan exposure in a non-dose responsive manner (Figure 2.2a), with a 25-32% decrease for all dose groups except 10 mg/kg/day. There was a significant main effect of dose [$F(5, 105)=7.34$, $p<0.0001$], but no main effect of study block ($p<0.05$), or dose-by study block interaction ($p>0.05$). PROD activity was significantly elevated in a dose-dependent manner (2.2b). The 100, 300, and 1000 mg/kg/day groups were increased by 249, 814, and 937 percent of control, respectively.

These conclusions are supported by a main effects of dose [$F(5,104)=29.95$, $p<0.0001$] and a dose-by study block interaction [$F(7,104)=4.86$, $p<0.0001$]. The interaction of study block and dose was due to a higher baseline activity in block 3 and a slightly greater induction compared to study blocks 1 and 2. Note that there was significant induction of PROD in all three study blocks. Mean contrast tests revealed significant effects in the 300 and 1000 mg/kg/day group ($p<0.05$). Using a BMR equivalent to a 50% increase in PROD activity, a Hill model fit to the data predicted a BMD of 70.8 mg/kg/day, with a 95% lower confidence limit of 48.6 mg/kg/day. The increased EROD (22-fold) and PROD (3.5-fold) activity in the positive control were consistent with laboratory historical control data.

Expression levels of hepatic microsomal CYP mRNA were differentially altered by triclosan exposure (Figure 2.3). There was no significant effect of triclosan on relative mRNA expression of *Cyp1a1*. *Cyp2b1/2* and *Cyp3a1/23* increased in a dose-dependent manner. There were main effects of dose for both *Cyp2b1/2* [$F(4,35)=3.30$, $p<0.0214$] and *Cyp3a1/23* [$F(4, 35)=9.52$, $p<0.0001$]. Mean contrast tests revealed significant increases of *Cyp2b1/2* mRNA of 2-fold at 300 mg/kg/day only. *Cyp3a1/23* was significantly increased 2.5- and 4.1-fold in the 100 and 300 mg/kg/day groups, respectively. Using a BMR equivalent to 20% increase in mRNA expression, the BMD and BMDL for *Cyp2b2* were 45.3 mg/kg/day and 24.0 mg/kg/day, and the BMD and BMDL for *Cyp3a1/23* were 62.3 and 7.3 mg/kg/day, respectively. The *Cyp2b1/2* expression data was best fit with a linear model, whereas the Hill model provided the best fit for the *Cyp3a1/23* expression data.

T4-glucuronidation activity increased with triclosan treatment (Figure 2.4). There were main effects of dose [$F(5,104)=2.38$, $p<0.0435$] and block [$F(2,104)=5.20$, $p<0.0070$], but no dose-by study block interaction ($p<0.05$). Mean contrast testing revealed a significant 82% increase at 1000 mg/kg/day. BMDS did not fit the data using linear, Hill, polynomial,

or exponential models.

Triclosan exposure induced isoform-specific increases in UGT and SULT mRNA expression (Figure 2.5 and 2.6). There was a main effect of dose on *Ugt1a1* mRNA expression [$F(4, 35)=5.98$, $p<0.0009$], but no significant effects on *Ugt1a6* and *Ugt2b5*. Mean contrast tests revealed significant 1.7- and 2.3-fold increases at 100 and 300 mg/kg/day, respectively. Using a BMR equivalent to a 20% increase in mRNA expression, the BMD and BMDL were 22.7 and 6.4 mg/kg/d, respectively. Triclosan increased *Sult1c1* mRNA expression [$F(4, 35)=6.34$, $p<0.0006$] approximately 2.5-fold in the 100 and 300 mg/kg/day groups (Figure 2.6). The significant main effect of triclosan on *Sult1b1* mRNA expression [$F(4, 34)=4.63$, $p<0.0043$], was caused by small but significant decreases at 30 and 100 mg/kg/day ($p<0.05$). Linear, Hill, polynomial, and exponential models failed to significantly fit the mRNA expression data for *Sult1b1* and *Sult1c1*.

Triclosan treatment did not significantly alter mRNA expression of any of the measured hepatic transporters: *Oatp1a1 (Oatp1), Oatp1a4 (Oatp2), Mrp2, or Mdr1b* (Figure 2.7).

5. Discussion

The current work tested the hypothesis that triclosan decreased serum T4 via upregulation of hepatic catabolism and altered expression of cellular transport proteins. Consistent with this hypothesis we report here that triclosan upregulates both mRNA expression and activity of some Phase I and II hepatic enzymes. Contradictory to this hypothesis was the lack of effects on any measured hepatic cellular transporters. Furthermore, the pattern of effects on hepatic mRNA for CYP and UGT isoforms suggests that the initiating event in the mode-of-action for triclosan-induced hypothyroxinemia is activation of hepatic CAR and PXR receptors.

Consistent with previous reports, oral exposure to triclosan produced hypothyroxinemia. Dose-responsive T4 decreases have been observed following both 4-day (Crofton et al. 2007) and 31-day exposures (Zorrilla et al. 2009). Effective doses that reduced T4 by 20% in the four-day exposures used in this work (BMD = 99.4 mg/kg/day) were higher compared to the smaller four-day assay data set published previously by this laboratory (BMD=69.7 mg/kg/day) (Crofton et al. 2007). The effective dose that reduced T4 by 20% following a 31-day triclosan exposure (BMD = 14.5 mg/kg/day) was much lower (Zorrilla et al. 2009) than the effective doses in the current study, likely due to the differences in exposure duration, as well as the sex, age, and strain of rats used. Strain may be a particularly important uncertainty in directly comparing studies of thyroid disruption, as background levels of thyroid hormones and metabolic responses may differ between strains (Lecureux et al. 2009).

T3 and TSH were also assessed to more fully characterize the impact of triclosan on circulating thyroid and pituitary hormones. The decreases (12-25%) in T3 observed at 300 and 1000 mg/kg/day were consistent with the previous reports that T3 was decreased approximately 20% after 31-days of 200 mg/kg/day triclosan treatment (Zorrilla et al. 2009). No change in TSH with triclosan treatment is also consistent with a previous report (Zorrilla et al. 2009). Other chemicals including polychlorinated biphenyls and 3-methylcholanthrene have significantly decreased T4 with no effects on TSH (Hood et al. 1999, Liu et al. 1995); this phenomenon of decreased T4 without compensatory TSH increase, while not well understood, has been hypothesized to result from a lack of induction of T3-glucuronidation (Barter & Klaassen 1994, Hood & Klaassen 2000).

Triclosan exposure increased enzymatic and mRNA markers of activity and expression of *Cyp2b1/2* and *Cyp3a1/23*, suggesting that triclosan may activate nuclear

receptors CAR and/or PXR. Increased PROD activity implies increased Cyp2b1/2 protein. This is consistent with previous *in vitro* and *in vivo* observations of triclosan-induced PROD activity and Cyp2b1/2 protein (Hanioka et al. 1997, Hanioka et al. 1996, Jinno et al. 1997, Zorrilla et al. 2009). Zorrilla *et al.* also found PROD activity increases *in vivo* following 31-day exposures (BMD =14.31 mg/kg/day)(Burke et al. 1994), compared to 70.8 mg/kg/day, obtained in the present study. Relative mRNA expression of *Cyp2b1/2* and *Cyp3a1/23* increased significantly. This suggests that triclosan may be capable of interacting with both CAR and PXR, consistent with activation of PXR *in vitro* in a human (HuH7 culture) receptor reporter assay (Jacobs et al. 2005). Currently, there are no published reports of triclosan activity toward CAR. However, our observations of increased Cyp2b1/2 activity and expression indicate potential CAR activation (Sueyoshi et al. 1999, Qatanani et al. 2005).

EROD activity decreased slightly, and was not dose-responsive, corresponding well to the lack of significant changes to *Cyp1a1* mRNA expression; collectively these data imply that AhR activation is not a key step in this mode-of-action. No constitutive expression of Cyp1a1 protein in rat liver and no induction of *Cyp1a1* mRNA with triclosan treatment suggest that these EROD decreases do not reflect Cyp1a1 activity inhibition, but rather the inhibition of one or more of several CYPs, including Cyp2c11 (Burke et al. 1994). Previous work with rat liver microsomes demonstrated that triclosan competitively inhibited EROD induction by 3-methylcholanthrene, a prototypical microsomal enzyme inducer of EROD and Cyp1a1 activities (Hanioka et al. 1996). However, the failure of triclosan to induce EROD is inconsistent with a report of triclosan agonism of AhR; triclosan activated AhR to 40% of the level of activation of TCDD, and inhibited TCDD activation by 30%, suggesting a competitive interaction (Ahn et al. 2008).

Importantly, the lack of *Cyp1a1* induction demonstrates that the minor dioxin contaminates found in the triclosan sample used in this study, 2,8,-dichlorodibenzodioxin and 2,4,8-trichlorodibenzodioxin, did not induce AhR-mediated effects on Phase I and Phase II hepatic enzymes. 2,8-dichlorodibenzodioxin is a very weak AhR agonist *in vitro*, and failed to induce EROD in vivo (Mason & Safe 1986).

Phase II glucuronidation and sulfation are upregulated by triclosan treatment, suggesting triclosan treatment increases hepatic T4 conjugation. Observations of a two-fold increase in glucuronidation activity of T4 and a 2.2-fold increase in *Ugt1a1* mRNA expression suggest small increases in T4-UGT activity result from triclosan exposure. Increases in glucuronidation result in increased biliary excretion of conjugated hormone (Vansell & Klaassen 2002, Vansell & Klaassen 2001, Liu et al. 1995, Barter & Klaassen 1994). Previous work in this laboratory and others has found that UGT activity may not have a clear linear relationship with T4 concentrations (Craft et al. 2002, Hood & Klaassen 2000). Another major uncertainty regarding the testing of UGT isoforms for mRNA expression levels is the choice of isoforms to test. This work tested isoforms responsible for hepatic conjugation of T4 in rat: Ugt1a1 and Ugt1a6 (Vansell & Klaassen 2002), as well as a marker of T3-glucuronidation, Ugt2b5 (Vansell & Klaassen 2002, Richardson et al. 2008). Whereas mRNA expression of *Ugt1a1* and *Ugt1a6* can be found in the liver, *Ugt1a7*, which also conjugates T4, appears to be constitutively expressed mainly in the intestine of rats (Shelby et al. 20032004), and inducible in liver by AhR agonists (Metz et al. 2000). Thus, *Ugt1a7* was excluded from analysis, as an amplification efficiency in the appropriate range could not be determined for analysis by qRT-PCR. No changes in expression were observed for *Ugt1a6* or *Ugt2b5*. In comparing the T4-UGT activity and mRNA expression data, it appears likely that the approximately 2-fold increase in T4- UGT activity may be attributed

to increases observed in *Ugt1a1* mRNA expression.

While increased activity and expression of UGT isoforms supports the overall hypothesis that triclosan treatment induces glucuronidation of TH, these data do not confirm a particular nuclear receptor pathway, as CAR, PXR, and the glucocorticoid receptor (GR), all transcriptionally regulate isoform *Ugt1a1* (Sugatani et al. 2005). *Ugt1a6* is more exclusively controlled by the AhR (Auyeung et al. 2003); the lack of expression change for *Ugt1a6* complements the unchanged expression of *Cyp1a1* and EROD activity. This is consistent with the lack of AhR activation by triclosan and the lack of an AhR contribution to the endpoints measured in the current work.

Triclosan exposure increased sulfotransferase *Sult1c1* mRNA expression 2.6-fold, indicating that increased sulfation and biliary excretion of T4 may occur. Sulfation deactivates T4 and increases its biliary excretion via enhancement of inner ring deiodination and blockage of outer ring deiodination for reactivation (Kester et al. 2003). Triclosan noncompetitively inhibits the *in vitro* human hepatic microsomal sulfation of 3-hydroxybenzo(a)pyrene (3-OH-BaP), bisphenol A (BPA), p-nitrophenol, and acetaminophen with IC_{50} concentrations in the low micromolar concentration range; competitive inhibition of the glucuronidation of 3-OH-BaP, BPA and acetaminophen was also observed (Wang et al. 2004). Thus, triclosan may exert an inhibitory action on SULT and UGT catabolic enzymes, affecting the overall activity profile of UGTs and SULTs *in vivo*. An uncertainty in our analysis is choice of sulfotransferases to test; sulfation of thyroid hormones is catalyzed primarily by Sult1b1 and Sult1c1 in the rat, though the preferred substrate for these enzymes is actually not the physiologically relevant thyroid hormone (T3), but rather a T3 metabolite (T2) (Kester et al. 2003). Any future use of this data in extrapolation to potential human effects is complicated by the sex dependence of *Sult1c1* expression in rats (Wong et

al. 2005), which signals a potentially sexually dimorphic response to triclosan with respect to sulfotransferase expression changes. Male-dominant expression of *Sult1c1* reflects sex-dependent T3 sulfation in rats, which is not known to occur in humans (Kaptein et al. 1997). Our observations report mRNA expression changes in female rats; however, the major change noted in sulfotransferases activity for triclosan treatment groups was up-regulation of *Sult1c1*, which is expressed predominantly in male rat liver, kidney, and intestine; expression of *Sult1b1* is equivalent between rat sexes (Kester et al. 2003).

No statistically significant changes were observed in the mRNA expression of hepatic transporters, including *Oatp1a1, Oatp1a4, Mrp2,* and *Mdr1*. These particular transporters were chosen for analysis based on their functional activity and affinity for iodothyronines (Wong et al. 2005), as well as several previous literature reports of microsomal enzyme inducer effects on these transporters (Cheng et al. 2005, Kretschmer & Baldwin 2005, Wong et al. 2005, Klaassen & Slitt 2005, Johnson & Klaassen 2002b). These mRNA expression results suggest that transporter expression changes do not contribute to the observed T4 decreases. However, these results do not exclude the possibility that hepatic transporter protein activity is altered by triclosan treatment via receptor-mediated post-translational modifications (Cherrington et al. 2002). The mRNA expression of *Mrp2* may not be indicative of the *in vivo* protein expression, as PXR ligands that induce Cyp3a have been shown to increase Mrp2 protein without increasing *Mrp2* expression (Johnson & Klaassen 2002a). Other PXR ligands, including DMP-904 (Wong et al. 2005), PCN, and spironolactone (Cheng et al. 2005), and CAR ligands including phenobarbital (Cherrington et al. 2002) have previously demonstrated effects on the regulation of hepatic transporter mRNA expression. Further characterization of the hepatic transporter activity of the liver in response to triclosan treatment will be necessary to conclude definitively that transporters are

or are not involved in triclosan-induced hypothyroxinemia.

This work suggests that triclosan triclosan upregulates Phase II glucuronidation and sulfation, and that this increased catabolism may be at least partially responsible for the triclosan-induced hypothyroxinemia observed. This is consistent with other microsomal enzyme inducers including phenobarbital, polychlorinated biphenyls, pregnenolone-16α-carbonitrile, dioxins, and others (Liu et al. 1995, Schuur et al. 1998). Correlation plots (not shown) of T4 concentration versus UGT activity, and *Ugt1a1* and *Sult1c1* expression revealed significant linear model fit ($p<0.05$), but poor R-squared values (R^2=0.07-0.18), suggesting that other mechanisms contribute to the T4 decreases observed. Triclosan may exert a direct effect on the thyroid and TH synthesis; however, preliminary data in *ex vivo* porcine thyroid peroxidase assays indicate no effect of triclosan (Dr. Michael Hornung, personal communication). Other mechanisms that could contribute to the observed hypothyroxinemia, including inhibition of iodide uptake, competition with TH serum binding proteins and upregulation of deiodinase activity, have not been assessed.

Extrapolation of the current findings to humans should be tempered by a number of uncertainties, including the dose range used herein, versus estimated human exposures. The BMDL for a 20% decrease in T4 calculated from the data presented is 65.6 mg/kg/day in a 4-day exposure scenario. This short-term exposure model is intended for use as a tool in exploring mechanisms of thyroid disruption, and does not model prolonged daily human exposure. The target human population of interest, due to the presence of triclosan in breast milk and its potential to disrupt thyroid hormone, and subsequent adverse impacts on neurological development, is the developing neonate and infant. Human infant daily oral exposure is estimated is to be 0.005 mg/kg/day (USEPA 2008). Thus, comparing our BMDL to this daily intake yields a margin of exposure of approximately 13,000-fold. Note that this

does not take into account uncertainty factors used in the extrapolation of data obtained in rats to human risk; pharmacodynamic and pharmacokinetic differences in triclosan metabolism between species may greatly impact the relative effects.

The present work confirms the conclusion that triclosan disrupts thyroxine and triiodothyronine in the rat. Furthermore, the effects of triclosan on hepatic mRNA and enzymatic activity point out important key events in one potential mode-of-action of triclosan-induced hypothyroxinemia in rats, and suggest that CAR/PXR activation, with subsequent upregulation of hepatic catabolism of T4, may be one mechanism that contributes to the observed hypothyroxinemia. In order to improve the ability to extrapolate these findings to humans, future research should characterize interspecies similarities and differences in these key events.

6. Supplementary Data

Supplemental Data Table (Appendix 1) lists the group mean, number of samples per group, and standard deviation for serum thyroid hormone concentrations; EROD, PROD, and UGT microsomal activities; and the qRT-PCR results, including fold change and ddCt data for each gene expression assay reported. Raw data files can be obtained by contacting the corresponding author.

7. Funding Information

KB Paul was funded through the EPA/UNC Toxicology Research Program, Training Agreement (CR833237) and also through the National Institute of Environmental Health Science Training Grant (T32-ES07126) during this work.

8. Acknowledgments

The authors would like to thank Dr. Joyce Royland for assistance in performing the RT-PCR assays, Dr. Leah Zorrilla, Angela Buckalew, and Ashley Murr for performing the TSH

assays. Drs. Susan Hester and Douglas Wolf are gratefully acknowledged for comments on an earlier version of this manuscript. We also thank Drs. James Plautz and Lisa Navarro for supplying analytical purity data on the triclosan used in this study.

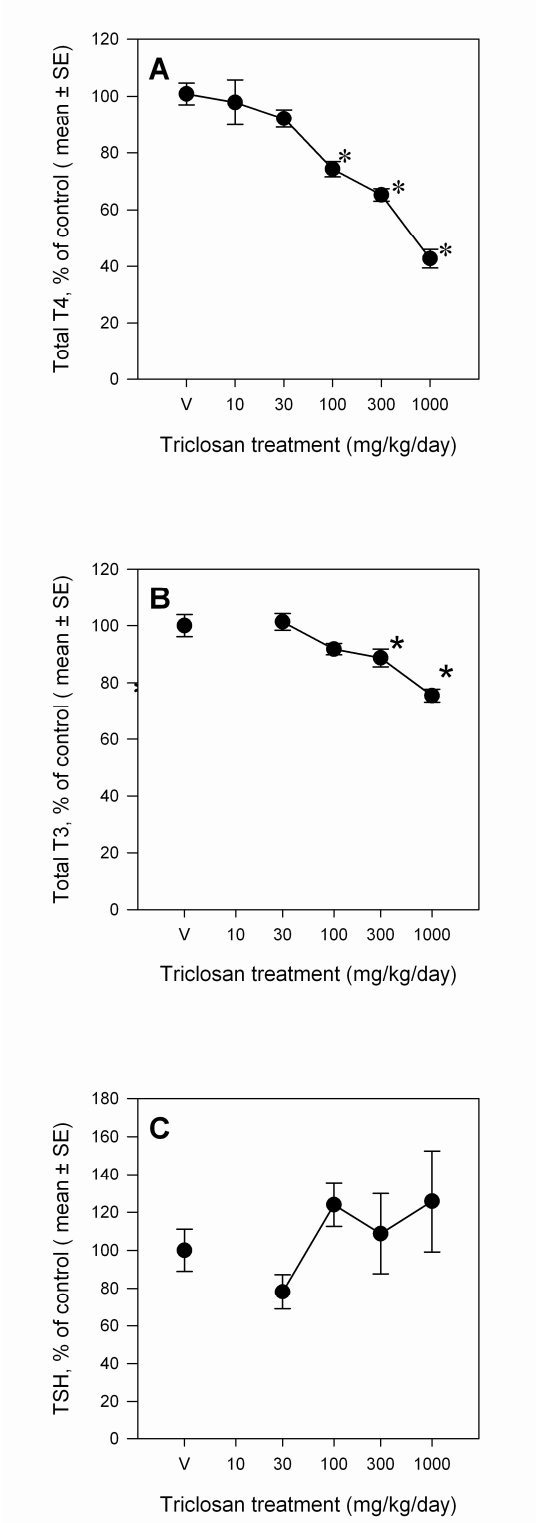


Figure 2.1. Triclosan decreases total serum T4 (A) and T3 (B), with no change in TSH (C), represented as group mean values (± SE) percent of vehicle control (V) for each study cohort. (V = vehicle control, corn oil; for T4: n = 24 for V, 30, 100, and 300 dose-groups, n = 16 for 1000 dose-group, n = 8 for 10 dose-group; for T3 and TSH: n=8 for V, 30, 100, 300, and 1000 dose-groups; * = significantly different from vehicle controls , p>0.05.

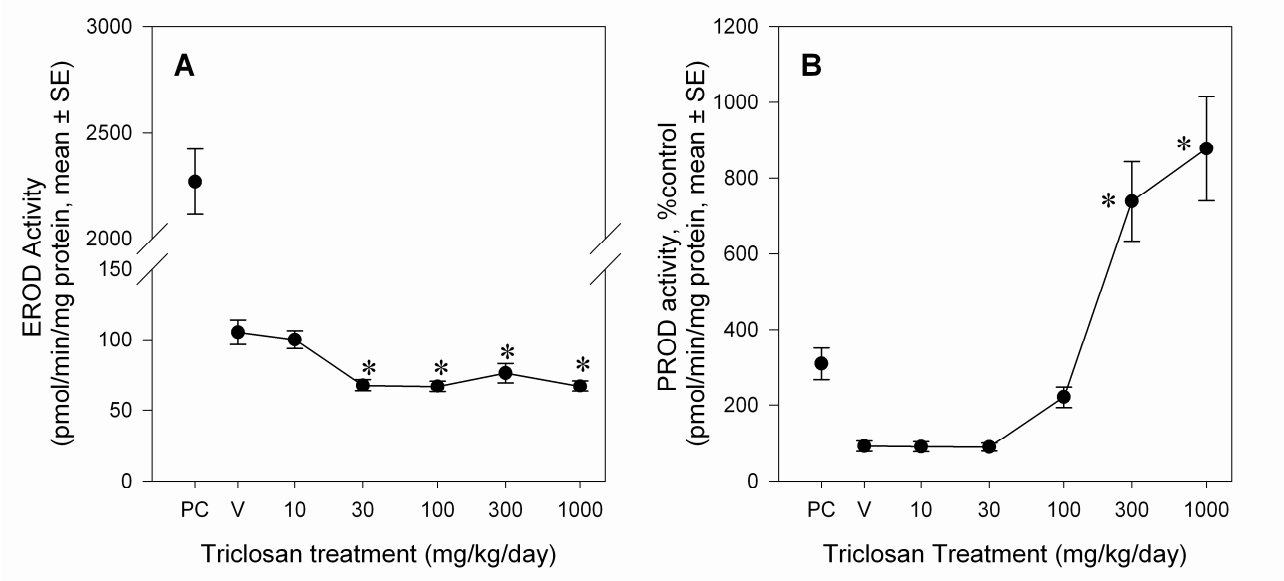


Figure 2.2. Dose-response curves for the effects of a 4-day exposure to triclosan on liver microsomal EROD activity (A), and PROD activity (B). Data are presented as group mean (± SE) percent of vehicle control (V) for each respective study block.(Activity = pmol resorufin/mg protein/min; V = vehicle control, corn oil; PC = positive control, single 10 µg/kg 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin dose and single 300 mg/kg Aroclor dose; n = 24 for V, 30, 100, and 300 dose-groups, n = 16 for 1000 dose-group, n = 8 for 10 dose-group; * = significantly different from vehicle control p>0.05).

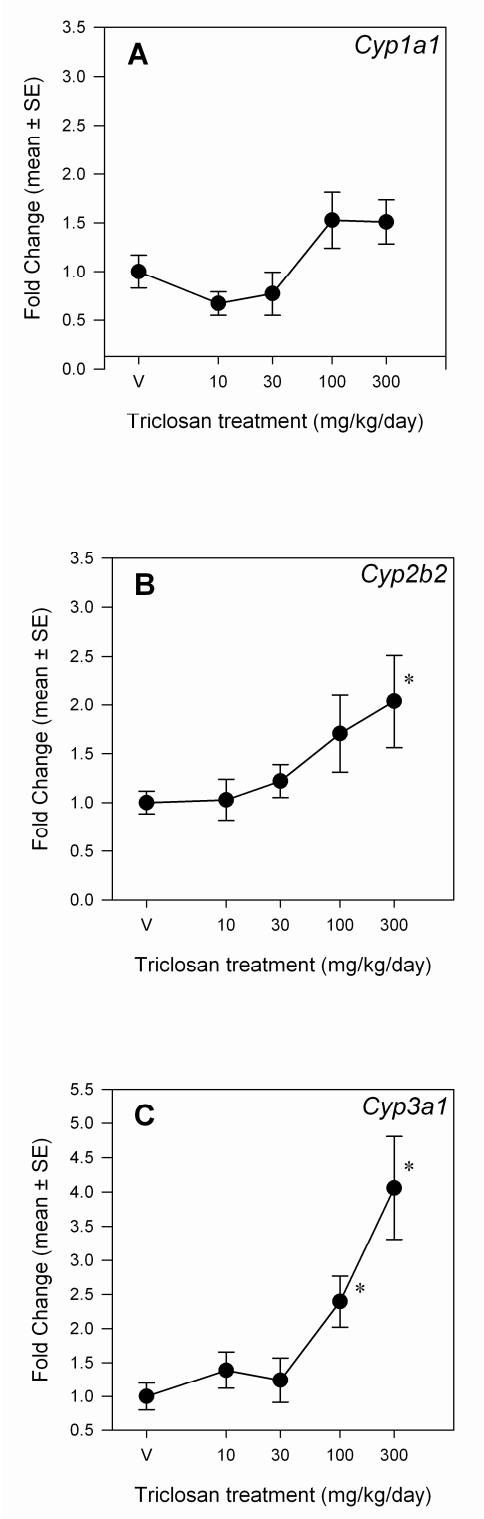


Figure 2.3. Dose-response curves for the effects of a 4-day exposure to triclosan on liver mRNA expression of CYP isoforms. Data are plotted as the mean (± SE) fold change ($2^{-\Delta\Delta C}t$) from control for the genes assayed: *Cyp1a1* (A), *Cyp2b2* (B), *Cyp3a1/23* (C). (V = vehicle control, corn oil; n = 8 per dose-group; * = significantly different from vehicle control $p>0.05$).

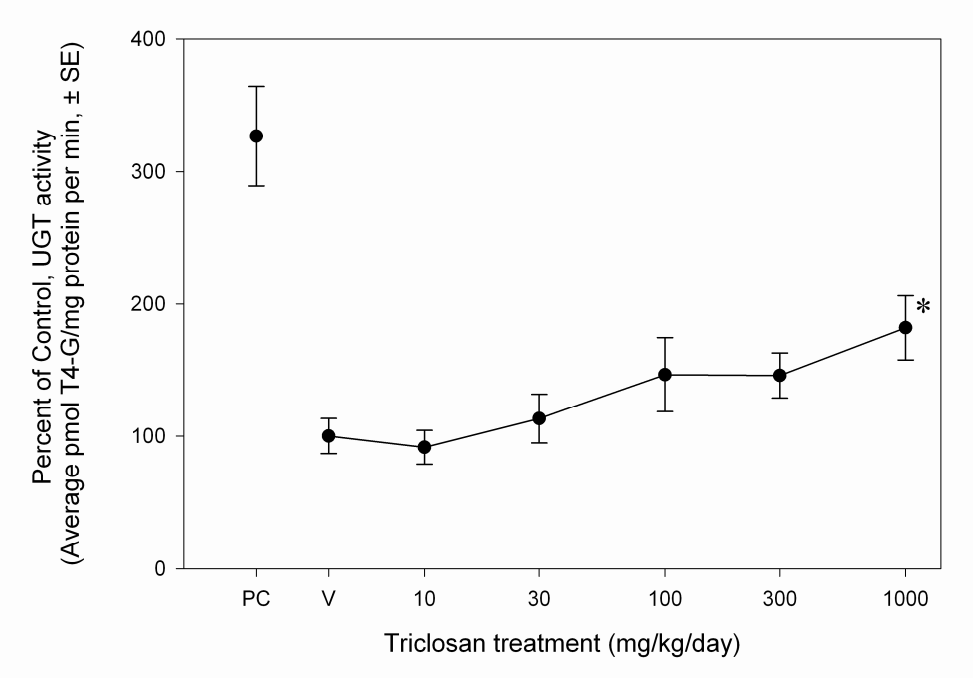


Figure 2.4. Dose-effect curve for the effects of a 4-day exposure to triclosan on liver microsomal UGT activity. Data are presented as group mean (± SE) percent of vehicle control (V) for each respective study block. (UGT activity = pmol T4-G/mg protein/min; V = vehicle control, corn oil; PC = positive control, single 10 µg/kg 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin dose and single 300 mg/kg Aroclor dose; n = 24 for V, 30, 100, and 300 dose-groups, n = 16 for 1000 dose-group, n = 8 for 10 dose-group; * = significantly different from vehicle control $p>0.05$).

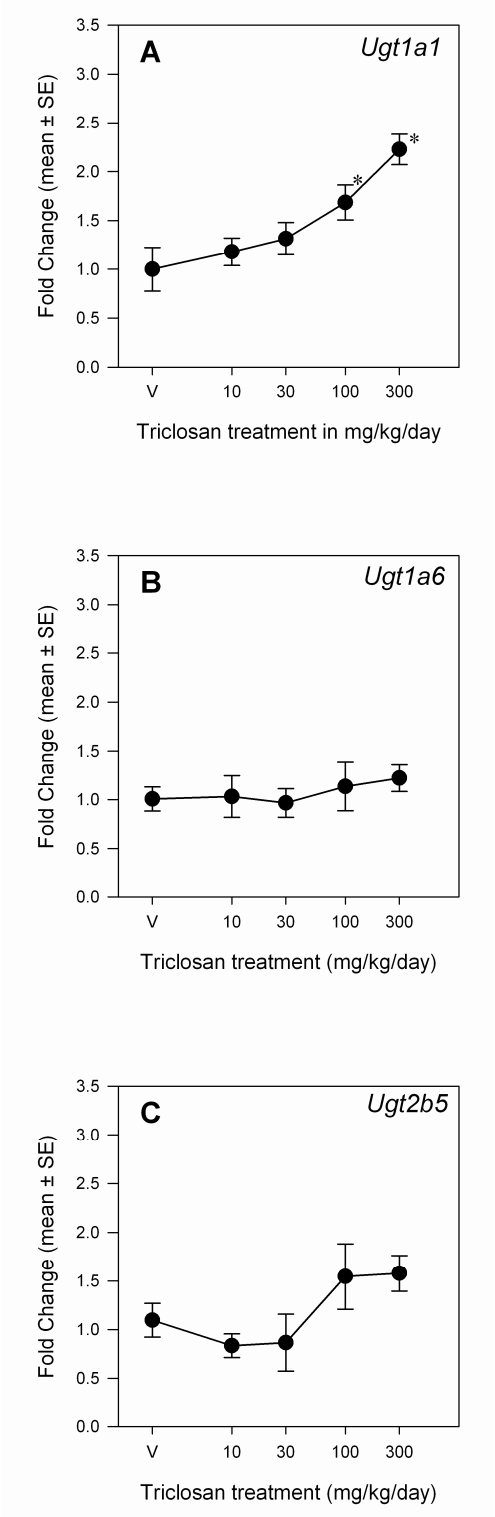


Figure 2.5. Dose-response curves for the effects of a 4-day exposure to triclosan on liver mRNA expression of UGT isoforms. Data are plotted as the mean (± SE) fold change ($2^{-\Delta\Delta C}t$) from control for the genes assayed: *Ugt1a1* (A), *Ugt1a6* (B), *Ugt2b5* (C). (V = vehicle control, corn oil; n =8 per dose-group; * = significantly different from vehicle control , $p>0.05$).

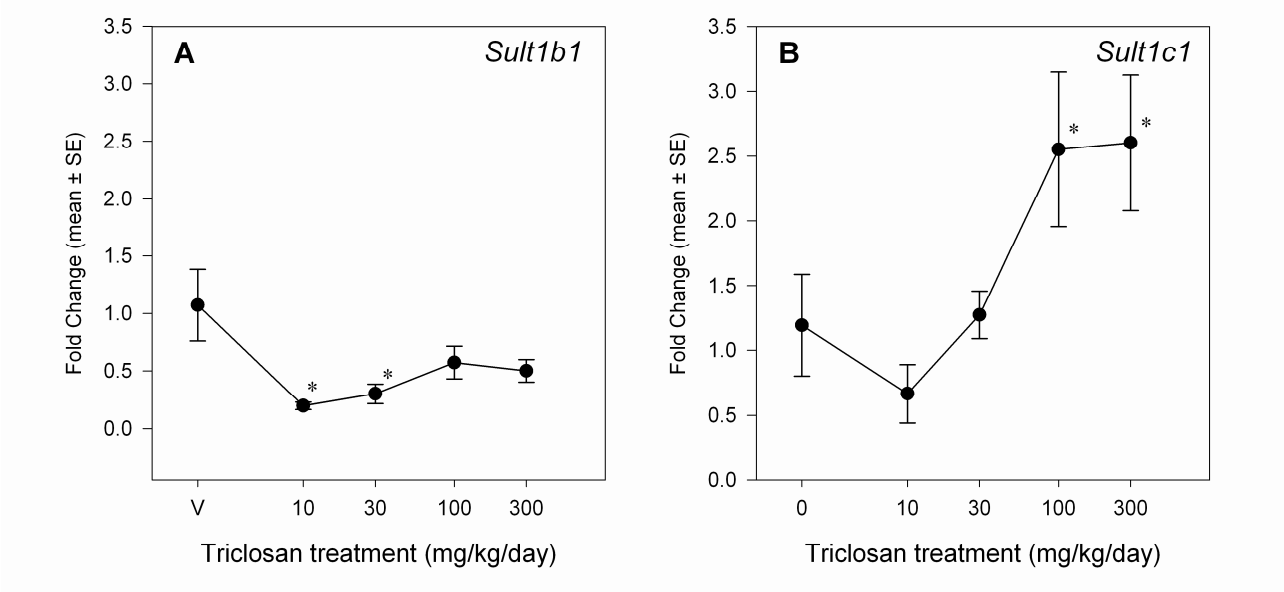


Figure 2.6. Dose-response curves for the effects of a 4-day exposure to triclosan on liver mRNA expression of SULT isoforms. Data are plotted as the mean (± SE) fold change ($2^{-\Delta\Delta C}t$) from control for the genes assayed: *Sult1b1* (A), *Sult1c1* (B). (V = vehicle control, corn oil; n =8 per dose group, with the exception of n = 7 for V dose group of *Sult1b1*; * = significantly different from vehicle control , p>0.05).

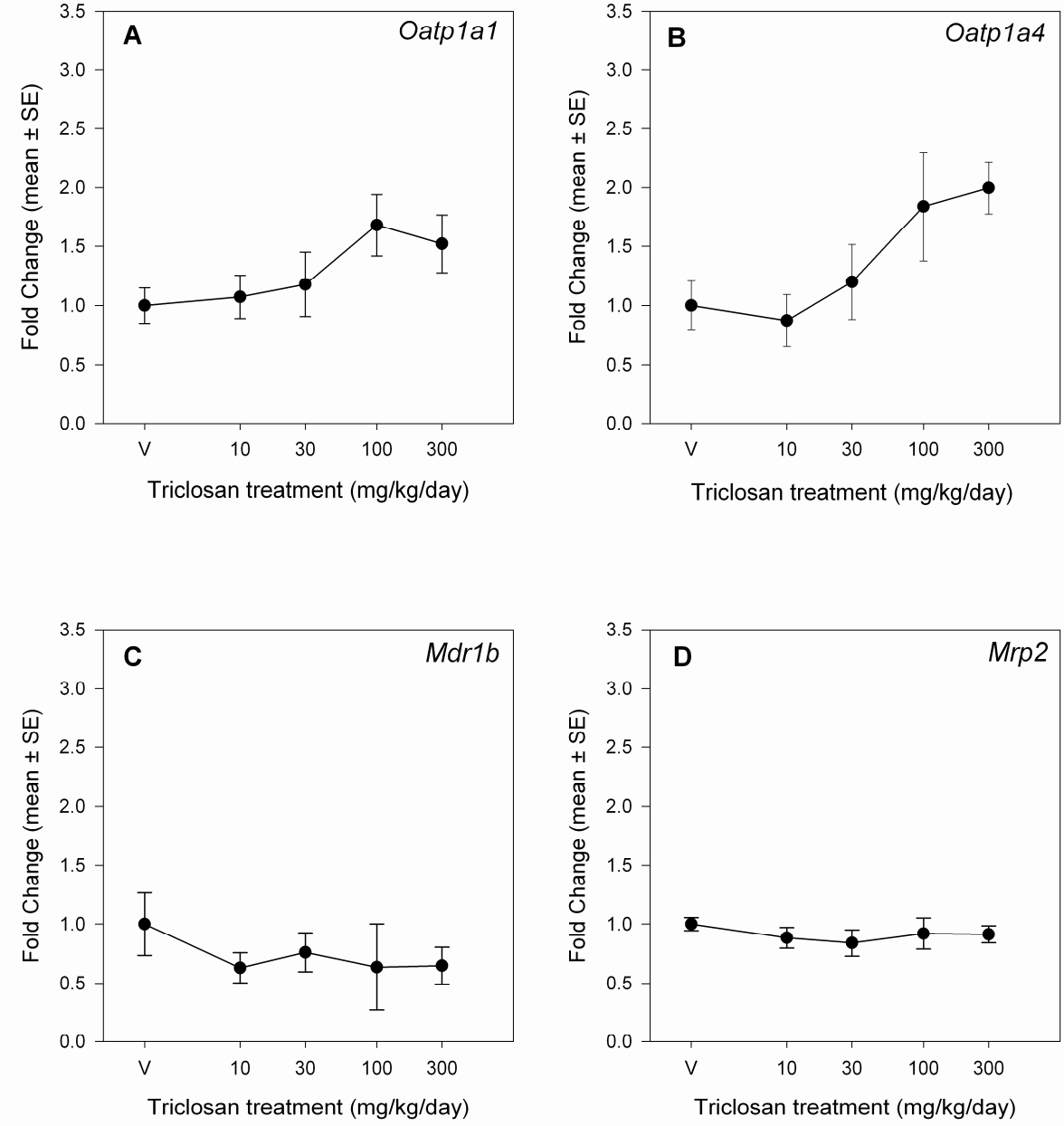


Figure 2.7. Dose-response curves for the effects of a 4-day exposure to triclosan on liver mRNA expression of transporters. Data are plotted as the mean (± SE) fold change ($2^{-\Delta\Delta C}t$) from control for the genes assayed: *Oatp1a1* (A), *Oatp1a4* (B), *Mdr1b* (C), *Mrp2* (D). (V = vehicle control, corn oil; n = 8 per dose-group).

Category	Gene	Abbreviation	Slope	R-Squared	Efficiency	Taqman Assay ID
Endogenous reference	Ribosomal protein S18	Rps18	-3.2493	0.9494	103.1%	Rn01428915_g1
Phase I markers	Cytochrome P450, family 1, subfamily a, polypeptide 1	Cyp1a1	-3.6424	0.8690	88.2%	Rn00487218_m1
	Cytochrome P450, family 2, subfamily b, polypeptide 2	Cyp2b1/2	-3.1757	0.9680	106.4%	Rn02783833_m1
	Cytochrome P450, subfamily 3A, polypeptide 1	Cyp3a1/23	-3.4391	0.9790	95.3%	Rn01640761_gH
Phase II markers	UDP-glycosyltransferase 1 family, polypeptide A1	Ugt1a1	-3.3699	0.9529	98.0%	Rn00754947_m1
	UDP-glycosyltransferase 1 family, polypeptide A6	Ugt1a6	-3.4287	0.9535	95.7%	Rn00756113_mH
	UDP-glucuronosyltransferase 2 family, member 5	Ugt2b5	-3.2953	0.8786	101.1%	Rn01777314_s1
	Sulfotransferase family 1B, member 1	Sult1b1	-3.2091	0.9143	104.9%	Rn00673872_m1
	Sulfotransferase family, cytosolic, 1C, member 1	Sult1c1	-3.2282	0.9747	104.1%	Rn00581955_m1
Phase III markers	Solute carrier organic anion transporter family, member 1a1	Oatp1a1 or Oatp1	-3.3182	1.0016	100.2	Rn00755148_m1
	Solute carrier organic anion transporter family, member 1a4	Oatp1a4 or Oatp2	-3.0310	1.1376	113.8%	Rn00756233_m1
	ATP-binding cassette, subfamily C (CFTR/MRP), member 2	Mrp2	-3.3033	1.0078	100.8%	Rn00563231_m1
	ATP-binding cassette, subfamily B (MDR/TAP), member 1B	Mdr1b	-3.6001	0.8957	89.6%	Rn00561753_m1

Table 2.1. Summary of Taqman Gene Expression Assays and Amplification Efficiencies. The endogenous reference gene Rps18 was used to standardize all of the gene expression assays. The slope presented is the slope value obtained from a plot of the threshold Ct (dRn) versus Log(RNA concentration), and R-squared value is the square of the correlation coefficient of the line obtained by the plot (Applied Biosystems Publication 127AP05-03).

Dose (mg/kg/d)	N per dose	Mean BW gain, % of control ± standard deviation	Mean liver weight, %control ± standard deviation	Mean liver-to-BW ratio, %control ± standard deviation
0	24	100 ± 16	100 ± 9.3	100 ± 4.9
10	8	102 ± 12	104 ± 18	105 ± 4.1
30	24	108 ± 16	103 ± 11	102 ± 4.7
100	24	105 ± 14	101 ± 12	101 ± 4.7
300	24	105 ± 24	105 ± 13	104 ± 5.2
1000	16	92 ± 20	112 ± 12*****	113 ± 4.9*****

Table 2.2. Doses, group sizes, and block-controlled body weight gain, liver weight, and liver-to-body-weight ratio with standard deviation values. Significant increases of nearly 12% and 13% occur in the 1000 mg/kg/day dose group for liver weight and liver-to-body-weight ratio, respectively (* = significantly different from vehicle control by ANOVA, $p>0.05$).

References

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J. & Sturve, J. (2002): Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere **46,** 1485-1489.

Ahn, K.C., Zhao, B., Chen, J., et al. (2008): In vitro biological activities of the anticmicrobials triclocarban, its analogues, and triclosan in bioassay screens: receptor-based bioassay screens. Environmental health perspectives.

Allmyr, M., Adolfsson-Erici, M., McLachlan, M.S. & Sandborgh-Englund, G. (2006): Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. The Science of the total environment **372,** 87-93.

Auyeung, D.J., Kessler, F.K. & Ritter, J.K. (2003): Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. Molecular pharmacology **63,** 119-127.

Barter, R.A. & Klaassen, C.D. (1994): Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. Toxicology and applied pharmacology **128,** 9-17.

Beetstra, J.B., van Engelen, J.G., Karels, P., et al. (1991): Thyroxine and 3,3',5-triiodothyronine are glucuronidated in rat liver by different uridine diphosphate-glucuronyltransferases. Endocrinology **128,** 741-746.

Bhargava, H.N. & Leonard, P.A. (1996): Triclosan: applications and safety. American journal of infection control **24,** 209-218.

Boas, M., Feldt-Rasmussen, U., Skakkebaek, N.E. & Main, K.M. (2006): Environmental chemicals and thyroid function. European journal of endocrinology / European Federation of Endocrine Societies **154,** 599-611.

Burke, M.D., Thompson, S., Weaver, R.J., Wolf, C.R. & Mayer, R.T. (1994): Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. Biochem Pharmacol **48,** 923-936.

Calafat, A.M., Ye, X., Wong, L.Y., Reidy, J.A. & Needham, L.L. (2008): Urinary concentrations of triclosan in the U.S. population: 2003-2004. Environmental health perspectives **116,** 303-307.

Capen, C.C. (1994): Mechanisms of chemical injury of thyroid gland. Progress in clinical and biological research **387,** 173-191.

Chen, J., Ahn, K.C., Gee, N.A., Gee, S.J., Hammock, B.D. & Lasley, B.L. (2007): Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. Toxicology and applied pharmacology **221,** 278-284.

Cheng, X., Maher, J., Dieter, M.Z. & Klaassen, C.D. (2005): Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. Drug metabolism and disposition: the biological fate of chemicals **33,** 1276-1282.

Cherrington, N.J., Hartley, D.P., Li, N., Johnson, D.R. & Klaassen, C.D. (2002): Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mrp1, 2, and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. The Journal of pharmacology and experimental therapeutics **300,** 97-104.

Chomczynski, P. & Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry **162,** 156-159.

Craft, E.S., DeVito, M.J. & Crofton, K.M. (2002): Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. Toxicol Sci **68,** 372-380.

Crofton, K.M. (2004): Developmental disruption of thyroid hormone: correlations with hearing dysfunction in rats. Risk Anal **24,** 1665-1671.

Crofton, K.M. (2008): Thyroid disrupting chemicals: mechanisms and mixtures. International journal of andrology **31,** 209-223.

Crofton, K.M., Paul, K.B., Hedge J.M. & M.J., D. (2007): Short-Term in Vivo Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine Environmental Toxicology and Pharmacology **24,** 194-197.

Crofton, K.M. & Zoeller, R.T. (2005): Mode of action: neurotoxicity induced by thyroid hormone disruption during development--hearing loss resulting from exposure to PHAHs. Crit Rev Toxicol **35,** 757-769.

Dayan, A.D. (2007): Risk assessment of triclosan [Irgasan((R))] in human breast milk. Food Chem Toxicol **45,** 125-129.

DeVito, M., Biegel, L., Brouwer, A., et al. (1999): Screening methods for thyroid hormone disruptors. Environmental health perspectives **107,** 407-415.

DeVito, M.J., Maier, W.E., Diliberto, J.J. & Birnbaum, L.S. (1993): Comparative Ability of Various PCBs , PCDFs, and TCDD to INduce Cytochrome P450 1A1 and 1a2 Activity Following 4 Weeks of Treatment. Fundamental and Applied Toxicology **20,** 125-130.

Dunn, R.T., 2nd & Klaassen, C.D. (1998): Tissue-specific expression of rat sulfotransferase messenger RNAs. Drug metabolism and disposition: the biological fate of chemicals **26,** 598-604.

EPA, U. (2000): Benchmark Dose Technical Guidance Document External Review Draft. Risk Assessment Forum **http://www.epa.gov/ncea/pdfs/bmds/BMD-External_10_13_2000.pdf**.

Gee, R.H., Charles, A., Taylor, N. & Darbre, P.D. (2008): Oestrogenic and androgenic activity of triclosan in breast cancer cells. J Appl Toxicol **28,** 78-91.

Greenwood, F.C., Hunter, W.M. & Glover, J.S. (1963): The Preparation of I-131-Labelled Human Growth Hormone of High Specific Radioactivity. Biochem J **89,** 114-123.

Hanioka, N., Jinno, H., Nishimura, T. & Ando, M. (1997): Effect of 2,4,4'-trichloro-2'-hydroxydiphenyl ether on cytochrome P450 enzymes in the rat liver. Chemosphere **34,** 719-730.

Hanioka, N., Omae, E., Nishimura, T., et al. (1996): Interaction of 2,4,4'-trichloro-2'-hydroxydiphenyl ether with microsomal cytochrome P450-dependent monooxygenases in rat liver. Chemosphere **33,** 265-276.

Hill, R.N., Crisp, T.M., Hurley, P.M., Rosenthal, S.L. & Singh, D.V. (1998): Risk assessment of thyroid follicular cell tumors. Environmental health perspectives **106,** 447-457.

Hood, A., Hashmi, R. & Klaassen, C.D. (1999): Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. Toxicology and applied pharmacology **160,** 163-170.

Hood, A. & Klaassen, C.D. (2000): Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. Toxicol Sci **55,** 78-84.

Houtman, C.J., Van Oostveen, A.M., Brouwer, A., Lamoree, M.H. & Legler, J. (2004): Identification of estrogenic compounds in fish bile using bioassay-directed fractionation. Environmental science & technology **38,** 6415-6423.

Hovander, L., Malmberg, T., Athanasiadou, M., et al. (2002): Identification of hydroxylated PCB metabolites and other phenolic halogenated pollutants in human blood plasma. Archives of environmental contamination and toxicology **42,** 105-117.

Jacobs, M.N., Nolan, G.T. & Hood, S.R. (2005): Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicology and applied pharmacology **209,** 123-133.

Jinno, H., Hanioka, N., Onodera, S., Nishimura, T. & Ando, M. (1997): Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel. Xenobiotica; the fate of foreign compounds in biological systems **27,** 681-692.

Johnson, D.R. & Klaassen, C.D. (2002a): Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. Toxicol Sci **67,** 182-189.

Johnson, D.R. & Klaassen, C.D. (2002b): Role of rat multidrug resistance protein 2 in plasma and biliary disposition of dibromosulfophthalein after microsomal enzyme induction. Toxicology and applied pharmacology **180,** 56-63.

Kaptein, E., van Haasteren, G.A., Linkels, E., de Greef, W.J. & Visser, T.J. (1997): Characterization of iodothyronine sulfotransferase activity in rat liver. Endocrinology **138,** 5136-5143.

Kester, M.H., Kaptein, E., Roest, T.J., et al. (2003): Characterization of rat iodothyronine sulfotransferases. American journal of physiology **285,** E592-598.

Klaassen, C.D. & Slitt, A.L. (2005): Regulation of hepatic transporters by xenobiotic receptors. Current drug metabolism **6,** 309-328.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., et al. (2002): Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environmental science & technology **36,** 1202-1211.

Kretschmer, X.C. & Baldwin, W.S. (2005): CAR and PXR: xenosensors of endocrine disrupters? Chemico-biological interactions **155,** 111-128.

Lecureux, L., Dieter, M.Z., Nelson, D.M., et al. (2009): Hepatobiliary disposition of thyroid hormone in Mrp2-deficient TR- rats: reduced biliary excretion of thyroxine glucuronide does not prevent xenobiotic-induced hypothyroidism. Toxicol Sci **108,** 482-491.

Liu, J., Liu, Y., Barter, R.A. & Klaassen, C.D. (1995): Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. The Journal of pharmacology and experimental therapeutics **273,** 977-985.

Livak, K.J. & Schmittgen, T.D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif **25,** 402-408.

Mason, G. & Safe, S. (1986): Synthesis, biologic and toxic effects of the major 2,3,7,8-tetrachlorodibenzo-p-dioxin metabolites in the rat. Toxicology **41,** 153-159.

McClain, R.M. (1989): The significance of hepatic microsomal enzyme induction and altered thyroid function in rats: implications for thyroid gland neoplasia. Toxicol Pathol **17,** 294-306.

McClain, R.M., Levin, A.A., Posch, R. & Downing, J.C. (1989): The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicology and applied pharmacology **99,** 216-228.

McClanahan, S.F. & Bartizek, R.D. (2002): Effects of triclosan/copolymer dentifrice on dental plaque and gingivitis in a 3-month randomized controlled clinical trial: influence of baseline gingivitis on observed efficacy. The Journal of clinical dentistry **13,** 167-178.

Metz, R.P., Auyeung, D.J., Kessler, F.K. & Ritter, J.K. (2000): Involvement of hepatocyte nuclear factor 1 in the regulation of the UDP-glucuronosyltransferase 1A7 (UGT1A7) gene in rat hepatocytes. Molecular pharmacology **58,** 319-327.

Paxeus, N. (1996): Organic pollutants in effluents of large wastewater treatment plants in Sweden. Water research **30,** 1115-1122.

Pohjanvirta, R., Niittynen, M., Linden, J., Boutros, P.C., Moffat, I.D. & Okey, A.B. (2006): Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin-treated rats. Chemico-biological interactions **160,** 134-149.

Qatanani, M., Zhang, J. & Moore, D.D. (2005): Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. Endocrinology **146,** 995-1002.

Richardson, V.M., Staskal, D.F., Ross, D.G., Diliberto, J.J., DeVito, M.J. & Birnbaum, L.S. (2008): Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener. Toxicology and applied pharmacology **226,** 244-250.

Sandborgh-Englund, G., Adolfsson-Erici, M., Odham, G. & Ekstrand, J. (2006): Pharmacokinetics of triclosan following oral ingestion in humans. Journal of toxicology and environmental health **69,** 1861-1873.

Schlecht, C., Klammer, H., Wuttke, W. & Jarry, H. (2006): A dose-response study on the estrogenic activity of benzophenone-2 on various endpoints in the serum, pituitary and uterus of female rats. Arch Toxicol **80,** 656-661.

Schuur, A.G., Legger, F.F., van Meeteren, M.E., et al. (1998): In vitro inhibition of thyroid hormone sulfation by hydroxylated metabolites of halogenated aromatic hydrocarbons. Chemical research in toxicology **11,** 1075-1081.

Shelby, M.K., L. Q., Cherrington, N.J., C. N., Vansell, N.R. & Klaassen, C.D., M. O. (20032004): Tissue mRNA expression of the rat Triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferase and UDP-glucuronosyltransferase gene familyglucuronosyl transferase in human liver fractions. Drug metabolism and disposition: the biological fate of chemicals **3132,** 326-331162-331169.

Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P. & Negishi, M. (1999): The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. J Biol Chem **274,** 6043-6046.

Sugatani, J., Sueyoshi, T., Negishi, M. & Miwa, M. (2005): Regulation of the human UGT1A1 gene by nuclear receptors constitutive active/androstane receptor, pregnane X receptor, and glucocorticoid receptor. Methods in enzymology **400,** 92-104.

USEPA (2008): Reregistration Eligibility Decision for Triclosan. In: EPA 739-RO-8009 (Office of Prevention, P., and Toxic Substances, ed.), pp. 1-47.

Vansell, N.R. & Klaassen, C.D. (2001): Increased biliary excretion of thyroxine by microsomal enzyme inducers. Toxicology and applied pharmacology **176,** 187-194.

Vansell, N.R. & Klaassen, C.D. (2002): Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. Drug metabolism and disposition: the biological fate of chemicals **30,** 240-246.

Veldhoen, N., Skirrow, R.C., Osachoff, H., et al. (2006): The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. Aquatic toxicology (Amsterdam, Netherlands) **80,** 217-227.

Wang, L.Q., Falany, C.N. & James, M.O. (2004): Triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. Drug metabolism and disposition: the biological fate of chemicals **32,** 1162-1169.

Wang, L.Q. & James, M.O. (2006): Inhibition of sulfotransferases by xenobiotics. Current drug metabolism **7,** 83-104.

Wolff, M.S., Teitelbaum, S.L., Windham, G., et al. (2007): Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. Environmental health perspectives **115,** 116-121.

Wong, H., Lehman-McKeeman, L.D., Grubb, M.F., et al. (2005): Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. Toxicol Sci **84,** 232-242.

Zhou, T., Ross, D.G., DeVito, M.J. & Crofton, K.M. (2001): Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol Sci **61,** 76-82.

Zhou, T., Taylor, M.M., DeVito, M.J. & Crofton, K.M. (2002): Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. Toxicol Sci **66,** 105-116.

Zorrilla, L.M., Gibson, E.K., Jeffay, S.C., et al. (2009): The effects of triclosan on puberty and thyroid hormones in male Wistar rats. Toxicol Sci **107,** 56-64.

Developmental Triclosan Exposure Decreases Maternal and Neonatal Thyroxine in Rats

Chapter 3

Katie B. Paul[1], Joan M. Hedge[2], Michael J. DeVito[3], Kevin M. Crofton[1,2*]

[1]Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC; [2]Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, RTP, NC; [3]National Toxicology Program, National Institute of Environmental Health Sciences, RTP, NC.

*Correspondence: Kevin M. Crofton, Ph.D., Integrated Systems Toxicology Division, MD-B105-04, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; phone: 919-541-2672; email: crofton.kevin@epa.gov

1. Abstract

Disruption of maternal thyroid hormones during fetal development may result in irreversible neurological consequences in offspring. The current work tested the hypothesis that perinatal triclosan exposure of dams decreases thyroxine in dams and offspring prior to weaning. Pregnant Long-Evans rats received triclosan by oral gavage (0-300 mg/kg/day) in corn oil from gestational day (GD) 6 through postnatal day (PND) 21. Serum was obtained from pups on PND4, 14, and 21, and from dams on PND22. Serum T4 was reduced 31% in dams on PND22. In pups, a unique pattern of hypothyroxinemia was observed; serum T4 decreased 27% in PND4 pups with no significant reduction observed on PND14 or PND21. Comparable reductions of approximately 30% in serum T4 at 300 mg/kg/day for dams and PND4 neonates and a lack of effect at PND14 and PND21 suggest that toxicokinetic or toxicodynamic factors may have contributed to a reduced exposure or a reduced toxicological response during the lactation period.

2. Introduction

Proper regulation of thyroid hormones is critical to the development and maturation of the nervous system in many vertebrates, including mammals. Haddow *et al.* demonstrated that low maternal serum T4 during gestation correlated with 4-10 point IQ deficits in children (Haddow *et al.*, 1999). The adverse impact of subclinical T4 concentration decreases during human development is unequivocal (Kooistra *et al.*, 2006; Pop *et al.*, 2003; Pop *et al.*, 1999). The correlation between maternal and neonatal hypothyroxinemia and neuronal development is also well established in the rat, making it an appropriate model of developmental TH insufficiency (Howdeshell, 2002; Miller *et al.*, 2009; Rice *et al.*, 2000; Zoeller *et al.*, 2000). Hypothyroxinemia during critical periods of neurodevelopment has been shown to result in

permanent effects, including sensory and motor deficits (Anderson, 2008; Crofton *et al.*, 2005; Morreale de Escobar *et al.*, 2004). While the precise mechanistic cascade of events that lead from decreased serum hormone levels to adverse neurodevelopmental outcomes is not yet known, measurement of circulating T4 in dams and offspring serves as a qualitative predictor of neurodevelopmental deficits (Crofton, 2004; Miller et al., 2009).

Triclosan is a bacteriostatic agent used in a variety of commercial personal care products. Widespread use and environmental transport has led to its detection as contaminant in surface water and wastewater treatment plant effluents at ng/L concentrations (Chalew *et al.*, 2009; Kanda *et al.*, 2003; Kolpin *et al.*, 2002; Loraine *et al.*, 2006; McAvoy *et al.*, 2002; Waltman *et al.*, 2006), in wildlife samples including fish (Adolfsson-Erici *et al.*, 2002; Balmer *et al.*, 2004; Houtman *et al.*, 2004), and also in human tissues including serum, urine, and milk (Adolfsson-Erici et al., 2002; Allmyr *et al.*, 2006; Allmyr *et al.*, 2008; Calafat *et al.*, 2008; Hovander *et al.*, 2002; Wolff *et al.*, 2007). Triclosan is an endocrine disruptor, reported to exert weak estrogenic effects on frogs and fish (Foran *et al.*, 2000; Ishibashi *et al.*, 2004; Matsumura *et al.*, 2005; Raut *et al.*, 2009), and to disrupt thyroid-mediated development in frogs (Veldhoen *et al.*, 2006) . Recent research using juvenile and weanling rat exposure models has demonstrated that triclosan exposure decreases thyroid hormone concentrations in mammalian models (Crofton *et al.*, 2007; Paul *et al.*, 2010; Zorrilla *et al.*, 2009). The endocrinological effects of triclosan exposure during rat development are currently unknown. The finding that triclosan is present in human milk at concentrations up to 2100 µg/kg-lipid (Dayan, 2007) has raised concem due to the documented relationship between thyroid disruption during development and irreversible adverse neurological outcomes in children.

Susceptibility to triclosan-induced hypothyroxinemia may differ between life stages due to potential differences in the toxicodynamic and toxicokinetic responses to triclosan during pregnancy and development. The current study was designed to test the hypothesis that perinatal triclosan exposure will alter circulating thyroid hormone levels in pups during early postnatal development and in dams at the conclusion of lactation. As only dams received triclosan directly, this study models the exposure of human mothers during gestation and nursing, and the potential combined *in utero* and nursing exposures to offspring.

3. Methods

Animals. Time-pregnant Long-Evans female rats (n=40), approximately 80-90 days of age, were obtained from Charles River Laboratories Inc. (Raleigh, NC) on gestation day (GD) 1 (defined as the day after vaginal plugs were observed), and were allowed five days of acclimation in an American Association for Accreditation of Laboratory Animal Care International (AALAC) approved animal facility prior to initiation of treatment on GD6. Animals were housed individually in plastic hanging cages (45 cm x 24 cm x 20 cm), with heat sterilized pine shavings bedding (Northeastern Products Corp., Warrenton, NC). Colony rooms were maintained at 21 ± 2°C with 50 ± 10 % humidity on a photo-period of 12L:12D. Food (Purina Rodent Chow #5001, Barnes Supply Co., Durham, NC) and water were provided *ad libitum*. Tap water (Durham, NC water) was filtered through sand, then activated charcoal, and finally re-chlorinated to 4-5 ppm Cl^- before use in the animal facility. All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the US EPA.

Dams (n=10/treatment for 0, 30, 100, 300 mg/kg/day treatment groups) were orally exposed via gavage each day between 0800 and 1000 hr, with the exception of GD21, when chemical was not administered to animals if there were signs of parturition. On GD21, dams were checked for the number of pups delivered at 0800, 1000, 1200, and 1500 hrs, and pups were aged as postnatal day (PND) 0 on the date of birth. All pups born from within a 24 hr period were considered to be the same age. On PND4, 14, and 21, offspring were counted, sexed, and group-weighed by sex. Average pup weight by sex was calculated by dividing the group weight by the number of pups. On PND4, litters were culled to 8 pups per litter, with the exception that litters comprised of fewer than 8 pups were not culled. Eye opening, determined as at least one eye open, was monitored once daily from PND11-17.

Chemicals and treatment. Triclosan (5-chloro-2-(2,4-dichloro-phenoxy)phenol (CAS#3380-34-5; 98+ % pure) was obtained from Sigma-Aldrich Chemical Company (St Louis, MO, LOT#06415CD, Cat#524190-10G) and Ciba Grenzach GmbH (Germany, Lot#60023CL7). Mass spectrometry analysis revealed that the triclosan used was greater than 98.2% pure triclosan; the sample also contained 0.05% iso-triclosan, 0.12% 2,8-dichlorodibenzodioxin, and 0.1% 2,4,8-trichlorodibenzodioxin, but was free of biologically-active dioxin compounds. The dosing solutions (0, 30, 100, and 300 mg/mL) were prepared in corn oil (Sigma, Lot#117K0127), sonicated for 30 minutes, and stored in amber vials at room temperature. Solutions were prepared every 5-7 days. The 300 mg/kg/day dose partially precipitated within 24 hours and was therefore sonicated daily before use. All doses were mixed on a stir plate during the dosing period each morning. Dams were randomly assigned to treatment groups by counter-balancing body weights. Administered volume was 1.0 ml corn oil/kg body weight; body weights were recorded daily, and administered volumes

were adjusted daily by weight. Prior to the sacrifice of pups on PND4 (only culled pups), 14 and 21 (one pup per sex per litter), animals were moved to a holding room, dams were weighed and dosed, pups were weighed, and all animals were acclimated for a minimum of 30 min. Tissue collection was conducted between 0800 and 1200 hrs in an adjacent room with a separate air supply. The time of necropsy within the 4-hr period was balanced among dose groups to control for time-of day effects on thyroxine levels (Dohler *et al.*, 1979; Jordan *et al.*, 1980). Trunk blood was collected from one male and one female pup per litter and pooled into one tube. Blood from dams was collected after decapitation on PND22, 24 hr after the final dose. Blood was collected into serum separator tubes (Beckton Dickinson, 36-6154). Serum was obtained after clotting whole blood for 30 min on ice, followed by centrifugation at 1278x g at 4°C for 30 min. Serum samples were stored at -80°C until analysis.

Thyroxine assay. Serum total T4 was measured in duplicate by standard solid-phase Coat-A-Count radioimmunoassay (RIA) kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assay variation was assessed using the multivalent control module (Siemens Medical Solutions Diagnostics, Los Angeles, CA) to measure low, medium, and high total T4 values before and after measuring the experimental samples. The intra-assay CV ranged from 4 to 5%. The inter-assay CV for three separate assays conducted over a one year period was 4.9%. Total serum T4 was calculated as ng T4/mL serum.

Data analysis. Dam bodyweight data (total n=38, one dam in the high dose group died of unknown causes and one dam failed to deliver live pups) for prenatal and postnatal stages were analyzed separately using repeated measures ANOVAs (SAS 9.1, SAS Institute, Cary, NC), followed by mean contrast testing with Duncan's New Multiple Range Test ($p<0.05$),

with dose and animal as independent variables. All other data were analyzed by ANOVAs, with significant main effects followed by Duncan's New Multiple Range Test. Gestation length was defined as the period from GD0 to the day of birth. The viability index was calculated as the number of pups alive on PND0 divided by the number alive on PND4 per litter prior to culling. Sex ratio was calculated as the number of female pups divided by the number of male pups. Eye opening was calculated as mean percent of pups with at least one eye open in a litter for each treatment.

Benchmark dose (BMD) and lower-bound confidence limit (BMDL) estimates were determined using USEPA Benchmark Dose Software (BMDS Version 2.0beta) as previously described (Crofton et al., 2007; Zhou *et al.*, 2001; Zhou *et al.*, 2002). The benchmark response (BMR) (EPA, 2000) was set at a 20% decrease in thyroxine, reflecting previous use of this BMR in the literature (Crofton et al., 2007; Paul et al., 2010; Zhou et al., 2001; Zhou et al., 2002; Zorrilla et al., 2009). The BMD was calculated from a model fit to the data. The BMDL (lower-bound confidence limit) was calculated as the 95% lower confidence interval.

4. Results

Perinatal maternal triclosan exposure did not affect any reproductive parameters (Table 3.1), including: gestation length [$F(3,35) = 0.00$, $p<0.9998$], litter size [$F(3,35)=0.48$, $p<0.6949$], viability index [$F(3,34)=0$, $p=1$], or sex ratio [$F(3,34)=0.76$, $p<0.5228$]. Treatment did not elicit effects on the day of eye opening [$F(3,33)=0.09$, $p<0.9650$] (Table 1). Further, no gross terata were observed in any of the pups, and viability was unaffected by treatment.

No clinical signs of toxicity were observed in the dams or pups during the 36 days of triclosan treatment. There was a 7-10% decrease in body weight observed for dams in the

300 mg/kg/day treatment group from GD14 through GD20 that was not statistically different from control; there was no main effect of treatment [$F(3,34)=2.56$, $p<0.0715$], nor was there an interaction of prenatal-day and treatment ($F(42,63)=1.05$, $p<0.4264$). Dam body weights decreased by approximately 10% throughout the postnatal period (Figure 3.1). This observation is supported by a main effect of treatment on the body weight of dams [$F(3,32)=4.63$, $p<0.0084$] and no treatment by postnatal day interaction [$F(60,40)=0.86$, $p<0.7005$]. Mean contrast testing demonstrated a significant difference between the control and 300 mg/kg/day treatment ($p<0.05$). There were no effects of treatment on pup body weight, male or female, at ages PND4, PND14, or PND21 (data not shown).

Serum total T4 decreased 31% on PND22 in dams treated with 300 mg/kg/day (Figure 3.2) [$F(3,34)=4.36$, $p<0.0105$] (see Supplemental Data table, Appendix 2, for group statistics). The no-observed effect level (NOEL) for triclosan and T4 was 100 mg/kg/day. Although several BMD models fit the data, a second degree polynomial model had the lowest residual values and predicted a BMD of 229 mg/kg/day, with a 95% lower confidence limit of 104 mg/kg/d (Table 2.2).

Maternal exposure to triclosan reduced serum total T4 in neonates. This effect was restricted to pups on PND4, when there was a 27% decrease in the 300 mg/kg/day group relative to controls [$F(3,31)=3.57$, $p<0.0252$]. There were no effects of triclosan on pup serum T4 concentrations at PND14 or PND21; pups born to the 300 mg/kg/day dam treatment group had only non-significant decreases in T4 of 16% and 8% at PND14 and PND21, respectively. The NOEL for triclosan dose to the dam and T4 in PND4 pups was 100 mg/kg/day. A second degree polynomial model fit to the data predicts a BMD of 113 mg/kg/day, with a 95% lower confidence limit of 58 mg/kg/day (Table 1). Although several

BMD models fit the data appropriately, the second degree polynomial model yielded the lowest scaled residuals.

5. Discussion

Perinatal maternal exposure to triclosan resulted in hypothyroxinemia in dams and young neonates. The magnitude of the observed hypothyroxinemia is consistent with previous reports of decreased serum T4 in juvenile and weanling rats (Crofton et al., 2007; Paul et al., 2010; Zorrilla et al., 2009). Importantly, T4 decreases were only observed in dams and PND4 offspring with no effects observed in pups on PND14 or PND21, indicating a unique pattern of postnatal hypothyroxinemia. The highest dose of triclosan decreased dam body weights during the postnatal lactation period, but did not affect any of the other reproductive endpoints including pup survival, sex ratio, eye opening, or pup body weights.

Thyroxine reduction in both dams and PND4 neonates demonstrates that both pups, during early postnatal development, and dams are susceptible to triclosan-induced hypothyroxinemia. Oral maternal triclosan exposure produced equivalent effects in dams at PND22 and neonates at PND4. Decreases of approximately 30% in serum T4 at 300 mg/kg/day in dams and developmentally exposed neonates are consistent with the serum T4 changes and potency reported for weanling rats following a 4-day triclosan exposure (Crofton et al., 2007; Paul et al., 2010). Exposures of juvenile rats to triclosan for thirty-one days demonstrated a greater potency, with significantly decreased T4 found at 30 mg/kg/day (Zorrilla et al., 2009). The discrepancy between these studies is likely due to differences in a number of experimental variables, including: increased exposure duration, juvenile developmental stage, gender, and rat strain.

Perinatal maternal triclosan exposure resulted in decreased circulating serum T4 that was observed on PND4, but not observed on PND14 or PND21 (Figure 2.2). The lack of significant changes in serum total T4 during the later stages of lactation, at PND14 and PND21, was unexpected. A number of previous studies on a variety of chemicals, including polybrominated diphenyl ethers, polychlorinated biphenyls, and 2,3,7,8-tetrachlorodibenzodioxin (TCDD), have demonstrated that postnatal thyroid hormones are usually affected by perinatal (i.e., gestational and lactation) maternal xenobiotic exposure during the entirety of the postnatal lactation period (Crofton *et al.*, 2000; Goldey *et al.*, 1995; Morse *et al.*, 1993; Zhou et al., 2001; Zhou et al., 2002). In this study, the observed thyroxine decrease in pups on PND4, followed by recovery to controls levels by PND21, is a unique pattern of effects for perinatal maternal exposure to thyroid disrupting xenobiotics. Toxicokinetic and/or toxicodynamic factors may account for this observed postnatal pattern. The toxicokinetic distribution of triclosan may limit maternal transfer via milk, and thus exposure may diminish with increasing postnatal age. The effect of triclosan on PND4 pups could have resulted from transplacental exposure to triclosan. Previous work with Aroclor 1254 (Crofton et al., 2000), a commercial mixture of highly lipophilic PCBs, demonstrated that maternal prenatal-only exposure produced a similar pattern of effects on postnatal T4 levels in pups, i.e., a larger effect on PND4, followed by a return to control values by PND21. While triclosan is known to be present in human milk (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Dayan, 2007), there are no published data on the toxicokinetic distribution of triclosan in rats. Further work is required to address the hypothesis that pups receive less triclosan via lactation compared to placental transfer to the fetus. Alternatively, triclosan may not produce an equivalent toxicodynamic effect in neonates. This presupposes

that the hypothyroxinemia found in PND4 pups is due to the persistence of prenatal effects of triclosan that dissipate with increasing postnatal age. This hypothesis further suggests that triclosan does not up-regulate key hepatic catabolic mechanisms, i.e., glucuronidation or sulfation of thyroid hormones, responsible for thyroid disruption in weanling rats (Paul et al., 2010). While there are no published data on the effects of triclosan on early postnatal catabolic enzymes, data from a number of other studies of xenobiotics clearly demonstrate the inducibilty of hepatic microsomal enzymes in pups during the lactation period (Lucier *et al.*, 1979; Morse et al., 1993; Zhou et al., 2002). Testing this hypothesis will require measurement of hepatic Phase I and II enzymes in neonates following developmental exposures.

This study provides the first report of triclosan-induced hypothyroxinemia following perinatal developmental exposure. The observed postnatal pattern of hypothyroxinemia in pups was unique in that the early postnatal effects recovered to control levels by the end of weaning. This pattern suggests that toxicokinetic factors may have affected maternal disposition of triclosan into milk and thereby limited lactation exposure to pups, or that the triclosan may not have triggered the same toxicodynamic effects in offspring during the lactation period as in exposed weanlings or dams. These data demonstrate that the maternal exposure to triclosan impacts thyroid hormones during development in the rat. The relevance of these findings for humans and wildlife is tempered by both the relatively high doses used and possible species-specific metabolic and kinetic processes. Future research should characterize the dose of triclosan received by developing rats from placental and lactation exposure, as well as the hepatic catabolic responses of developing neonates to triclosan in order to explain the lack of effects of triclosan at later postnatal ages.

6. Acknowledgements

The authors would like to thank David Ross and Kateland Grant for assistance during animal sacrifice. Drs. Ram Ramabhadran, Sid Hunter, William Mundy, Tammy Stoker, Doug Wolf, and Leah Zorrilla are gratefully acknowledged for comments on an earlier version of this manuscript. Triclosan was a generous gift from Drs. James Plautz and Lisa Navarro of Ciba Specialty Chemicals. Raw data files can be obtained by contacting the corresponding author.

7. Funding

K.B. Paul was funded by a PhRMA Foundation Predoctoral Pharmacology/Toxicology Fellowship, the EPA/UNC Toxicology Research Program Training Agreement (CR833237), and the National Institute of Environmental Health Science Training Grant (T32-ES07126) during this work.

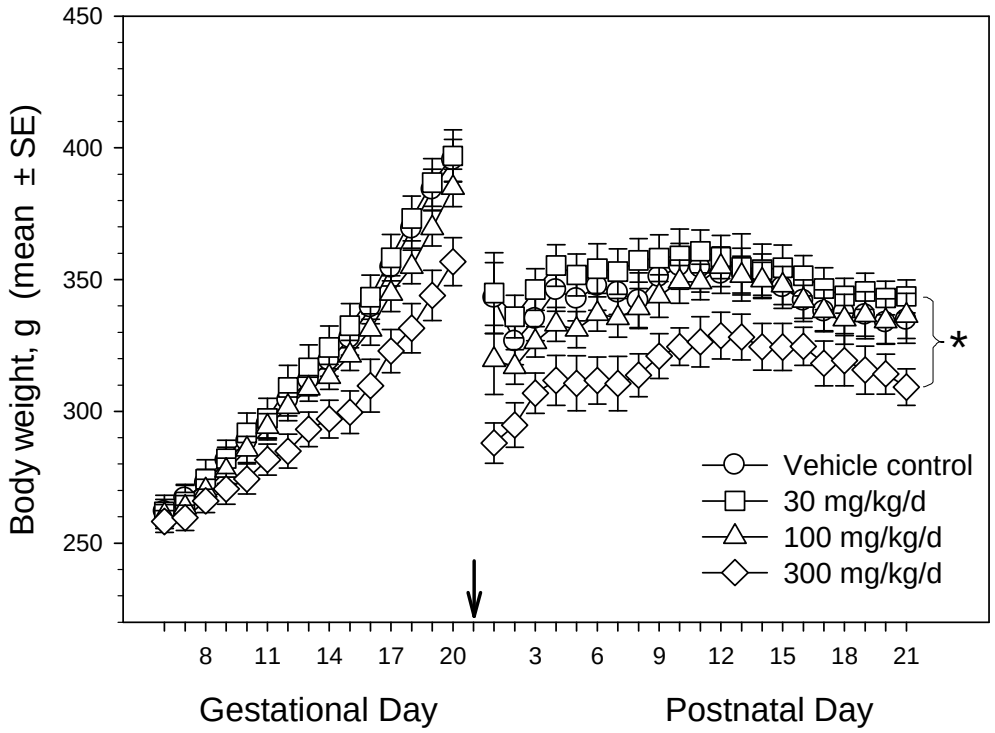


Figure 3.1. Dam body weight during the gestational and lactation periods. There is a statistically significant decrease of approximately 10% in the body weight of dams throughout the postnatal period for the 300 mg/kg/d dose group. [n = 10, 10, 9, 8 for 0, 30, 100 and 300 mg/kg/day, respectively; * = significantly main effect of treatment for postnatal ages, and group mean differences between vehicle and 300 mg/kg/d, p>0.05.]

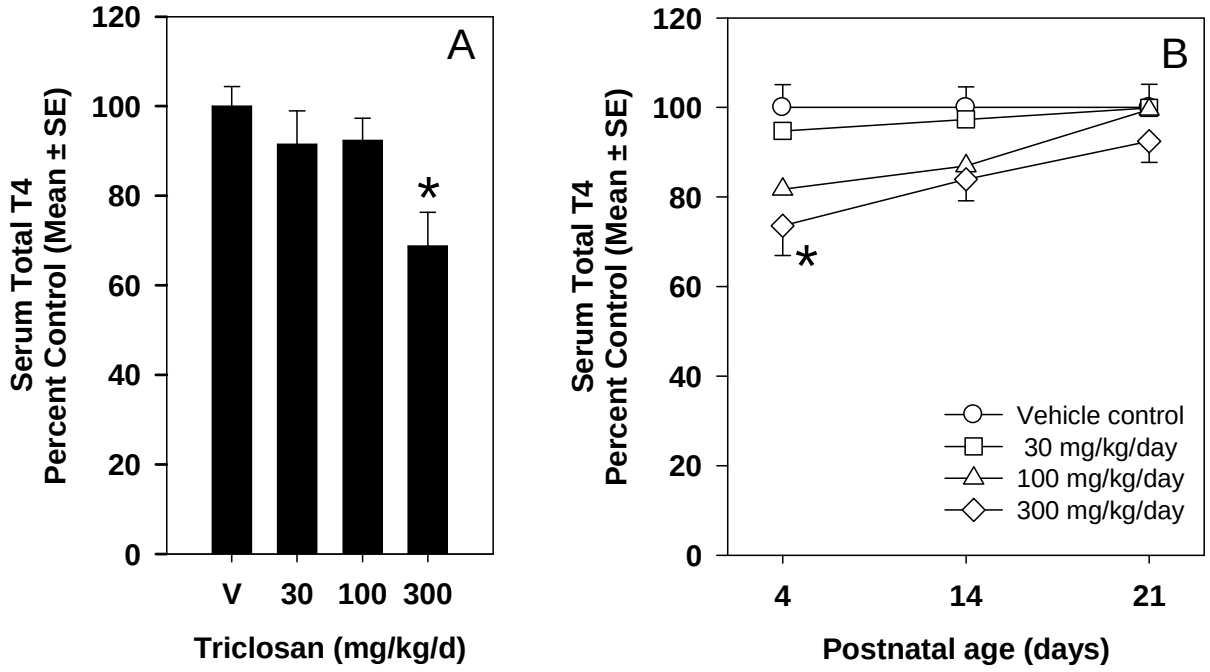


Figure 3.2. Percent of control serum total T4 for: A) Dams and B) Pups, by age and oral maternal dose (mean ± SE). [For Dams and PND21, n = 10, 10, 9, 8 for the 0, 30, 100 and 300 mg/kg/day, respectively; For PND14, n = 10, 10, 8, 8 for the 0, 30, 100 and 300 mg/kg/day, respectively; For PND4, n = 9, 9, 8, 8 for the 0, 30, 100 and 300 mg/kg/day, respectively; * = significantly different from vehicle control by ANOVA, p>0.05. Raw T4 values in ng/mL (mean ± SE) for the 0 mg/kg/d and 300 mg/kg/d groups, respectively, were: 49.6 ± 2.2 and 34.1 ± 3.7 for PND22 Dams; 9.63 ± 0.49 and 7.08 ± 0.64 for PND4 pups; 48.4 ± 2.2 40.6 ± 2.3 for PND14 pups; and 38.9 ± 2.0 and 36.0 ± 1.8 for PND21 pups.

Dose (mg/kg/day)	Gestation Length	Sex Ratio	Litter Size (PND 0)	Viability Index	% Eye Opening (PND)			
					13	14	15	16
0	21.1 ± 0.1	1.21 ± 0.25	12.8 ± 0.71	100.0 ± 0.0	0.0 ± 0.0	24.0 ± 7.2	91.7 ± 3.7	100 ± 0.0
30	21.1 ± 0.1	1.62 ± 0.44	11.8 ± 0.72	100.0 ± 0.0	0.0 ± 0.0	25.3 ± 7.1	91.7 ± 6.9	100 ± 0.0
100	21.1 ± 0.1	1.09 ± 0.16	11.5 ± 1.17	100.0 ± 0.0	0.0 ± 0.0	24.1 ± 8.4	98.1 ± 1.8	100 ± 0.0
300	21.1 ± 0.1	1.11 ± 0.12	11.1 ± 1.44	100.0 ± 0.0	0.0 ± 0.0	21.7 ± 8.1	97.9 ± 2.1	100 ± 0.0

Table 3.1. Gestation length, gender ratio, litter size, viability and eye opening (mean ± SE). None of the parameters shown were significantly changed by triclosan treatment. Gestation Length is reported in days; Gender Ratio is the number of females divided by the number of males; Litter Size at PND0 includes only live born pups; Viability Index is the littersize at PND4 divided by the live born pups at PND0 multiplied by 100%; % Eye Opening is the percent of pups in each litter with at least one eye open.

Age	NOEL	BMD	BMDL
Dam	100	229	104
PND4	100	113	58

Table 3.2. NOEL and BMD dose levels for T4 effects by age (mg/kg/day ± SE**).** US EPA Benchmark Dose Software (BMDS Version 2.0Beta) was used to determine the BMD and BMDL, with a 95% confidence limit, for the selected BMR = 20% reduction in serum T4.

References

Adolfsson-Erici, M., M. Pettersson, J. Parkkonen and J. Sturve (2002). Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**, 1485-1489.

Allmyr, M., M. Adolfsson-Erici, M. S. McLachlan and G. Sandborgh-Englund (2006). Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *The Science of the total environment* **372**, 87-93.

Allmyr, M., F. Harden, L. M. Toms, J. F. Mueller, M. S. McLachlan, M. Adolfsson-Erici and G. Sandborgh-Englund (2008). The influence of age and gender on triclosan concentrations in Australian human blood serum. *The Science of the total environment* **393**, 162-167.

Anderson, G. W. (2008). Thyroid hormone and cerebellar development. *Cerebellum* **7**, 60-74.

Balmer, M. E., T. Poiger, C. Droz, K. Romanin, P. A. Bergqvist, M. D. Muller and H. R. Buser (2004). Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland. *Environmental science & technology* **38**, 390-395.

Calafat, A. M., X. Ye, L. Y. Wong, J. A. Reidy and L. L. Needham (2008). Urinary concentrations of triclosan in the U.S. population: 2003-2004. *Environmental health perspectives* **116**, 303-307.

Chalew, T. E. and R. U. Halden (2009). Environmental Exposure of Aquatic and Terrestrial Biota to Triclosan and Triclocarban. *J Am Water Works Assoc* **45**, 4-13.

Crofton, K. M., P. R. Kodavanti, E. C. Derr-Yellin, A. C. Casey and L. S. Kehn (2000). PCBs, thyroid hormones, and ototoxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. *Toxicol Sci* **57**, 131-140.

Crofton, K. M. (2004). Developmental disruption of thyroid hormone: correlations with hearing dysfunction in rats. *Risk Anal* **24**, 1665-1671.

Crofton, K. M. and R. T. Zoeller (2005). Mode of action: neurotoxicity induced by thyroid hormone disruption during development--hearing loss resulting from exposure to PHAHs. *Critical reviews in toxicology* **35**, 757-769.

Crofton, K. M., K. B. Paul, Hedge J.M. and D. M.J. (2007). Short-Term in Vivo Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine *Environmental Toxicology and Pharmacology* **24**, 194-197.

Dayan, A. D. (2007). Risk assessment of triclosan [Irgasan((R))] in human breast milk. *Food Chem Toxicol* **45**, 125-129.

Dohler, K. D., C. C. Wong and A. von zur Muhlen (1979). The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. *Pharmacol Ther B* **5**, 305-318.

EPA, U. S. (2000). Benchmark Dose Technical Guidance Document External Review Draft. *Risk Assessment Forum* **http://www.epa.gov/ncea/pdfs/bmds/BMD-External_10_13_2000.pdf**.

Foran, C. M., E. R. Bennett and W. H. Benson (2000). Developmental evaluation of a potential non-steroidal estrogen: triclosan. *Marine environmental research* **50**, 153-156.

Goldey, E. S., L. S. Kehn, G. L. Rehnberg and K. M. Crofton (1995). Effects of developmental hypothyroidism on auditory and motor function in the rat. *Toxicology and applied pharmacology* **135**, 67-76.

Haddow, J. E., G. E. Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *The New England journal of medicine* **341**, 549-555.

Houtman, C. J., A. M. Van Oostveen, A. Brouwer, M. H. Lamoree and J. Legler (2004). Identification of estrogenic compounds in fish bile using bioassay-directed fractionation. *Environmental science & technology* **38**, 6415-6423.

Hovander, L., T. Malmberg, M. Athanasiadou, I. Athanassiadis, S. Rahm, A. Bergman and E. K. Wehler (2002). Identification of hydroxylated PCB metabolites and other phenolic halogenated pollutants in human blood plasma. *Archives of environmental contamination and toxicology* **42**, 105-117.

Howdeshell, K. L. (2002). A model of the development of the brain as a construct of the thyroid system. *Environmental health perspectives* **110 Suppl 3**, 337-348.

Ishibashi, H., N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao and K. Arizono (2004). Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. *Aquatic toxicology (Amsterdam, Netherlands)* **67**, 167-179.

Jordan, D., B. Rousset, F. Perrin, M. Fournier and J. Orgiazzi (1980). Evidence for circadian variations in serum thyrotropin, 3,5,3'-triiodothyronine, and thyroxine in the rat. *Endocrinology* **107**, 1245-1248.

Kanda, R., P. Griffin, H. A. James and J. Fothergill (2003). Pharmaceutical and personal care products in sewage treatment works. *J Environ Monit* **5**, 823-830.

Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber and H. T. Buxton (2002). Pharmaceuticals, hormones, and other organic wastewater

contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environmental science & technology* **36**, 1202-1211.

Kooistra, L., S. Crawford, A. L. van Baar, E. P. Brouwers and V. J. Pop (2006). Neonatal effects of maternal hypothyroxinemia during early pregnancy. *Pediatrics* **117**, 161-167.

Loraine, G. A. and M. E. Pettigrove (2006). Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. *Environmental science & technology* **40**, 687-695.

Lucier, G. W. and O. S. McDaniel (1979). Developmental toxicology of the halogenated aromatics: effects on enzyme development. *Ann N Y Acad Sci* **320**, 449-457.

Matsumura, N., H. Ishibashi, M. Hirano, Y. Nagao, N. Watanabe, H. Shiratsuchi, T. Kai, T. Nishimura, A. Kashiwagi and K. Arizono (2005). Effects of nonylphenol and triclosan on production of plasma vitellogenin and testosterone in male South African clawed frogs (Xenopus laevis). *Biological & pharmaceutical bulletin* **28**, 1748-1751.

McAvoy, D. C., B. Schatowitz, M. Jacob, A. Hauk and W. S. Eckhoff (2002). Measurement of triclosan in wastewater treatment systems. *Environmental toxicology and chemistry / SETAC* **21**, 1323-1329.

Miller, M. D., K. M. Crofton, D. C. Rice and R. T. Zoeller (2009). Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. *Environmental health perspectives* **117**, 1033-1041.

Morreale de Escobar, G., M. J. Obregon and F. Escobar del Rey (2004). Role of thyroid hormone during early brain development. *European journal of endocrinology / European Federation of Endocrine Societies* **151 Suppl 3**, U25-37.

Morse, D. C., D. Groen, M. Veerman, C. J. van Amerongen, H. B. Koeter, A. E. Smits van Prooije, T. J. Visser, J. H. Koeman and A. Brouwer (1993). Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. *Toxicology and applied pharmacology* **122**, 27-33.

Paul, K. B., J. M. Hedge, M. J. DeVito and K. M. Crofton (2010). Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol Sci* **113**, 367-379.

Pop, V. J., J. L. Kuijpens, A. L. van Baar, G. Verkerk, M. M. van Son, J. J. de Vijlder, T. Vulsma, W. M. Wiersinga, H. A. Drexhage and H. L. Vader (1999). Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clinical endocrinology* **50**, 149-155.

Pop, V. J., E. P. Brouwers, H. L. Vader, T. Vulsma, A. L. van Baar and J. J. de Vijlder (2003). Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clinical endocrinology* **59**, 282-288.

Raut, S. and R. Angus (2009). Assessment of short-term and long-term exposures of non-steroidal estrogen, triclosan in western male mosquitofish, Gambusia affinis. *Integr Comp Biol* **49**, E141-E141.

Rice, D. and S. Barone, Jr. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental health perspectives* **108 Suppl 3**, 511-533.

Veldhoen, N., R. C. Skirrow, H. Osachoff, H. Wigmore, D. J. Clapson, M. P. Gunderson, G. Van Aggelen and C. C. Helbing (2006). The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquatic toxicology (Amsterdam, Netherlands)* **80**, 217-227.

Waltman, E. L., B. J. Venables and W. T. Waller (2006). Triclosan in a north Texas wastewater treatment plant and the influent and effluent of an experimental constructed wetland. *Environmental toxicology and chemistry / SETAC* **25**, 367-372.

Wolff, M. S., S. L. Teitelbaum, G. Windham, S. M. Pinney, J. A. Britton, C. Chelimo, J. Godbold, F. Biro, L. H. Kushi, C. M. Pfeiffer and A. M. Calafat (2007). Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. *Environmental health perspectives* **115**, 116-121.

Zhou, T., D. G. Ross, M. J. DeVito and K. M. Crofton (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zhou, T., M. M. Taylor, M. J. DeVito and K. M. Crofton (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci* **66**, 105-116.

Zoeller, R. T., A. L. Dowling and A. A. Vas (2000). Developmental exposure to polychlorinated biphenyls exerts thyroid hormone-like effects on the expression of RC3/neurogranin and myelin basic protein messenger ribonucleic acids in the developing rat brain. *Endocrinology* **141**, 181-189.

Zorrilla, L. M., E. K. Gibson, S. C. Jeffay, K. M. Crofton, W. R. Setzer, R. L. Cooper and T. E. Stoker (2009). The effects of triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol Sci* **107**, 56-64.

Developmental triclosan exposure decreases maternal, fetal, and early neonatal thyroxine: A toxicodynamic and toxicokinetic analysis

Chapter 4

Katie B. Paul[1], Joan M. Hedge[2], Ruby Bansal[3], Tom Zoeller[3], Robert Peter[4], Michael J. DeVito[5], Kevin M. Crofton[2]

[1]University of North Carolina at Chapel Hill, Curriculum in Toxicology, CB 7270, Chapel Hill, NC 27599; [2]Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; [3]Department of Biology, University of Massachusetts, Amherst, MA 01003; [4]Intertek Expert Services, Ciba Inc., 4002 Basel, Switzerland; [5]National Toxicology Program, National Institute of Environmental Health Sciences Research, Triangle Park, North Carolina, USA

1. Abstract

Perinatal triclosan (TCS) exposure decreases serum maternal and early neonatal thyroxine (T4) in the rat, but the mode-of-action (MOA) for these effects, and the potential for these effects during gestation in addition to postnatally, is unknown. This work test the hypothesis that TCS decreases T4 via activation of the pregnane X and/or constitutive androstane receptors (PXR, CAR), resulting in up-regulation of hepatic catabolism of T4, and subsequent declines in circulating T4 and triiodothyronine (T3). Time-pregnant Long-Evans rats received TCS po (0-300 mg/kg/day) from gestational day (GD) 6 through weaning on postnatal day (PND) 21. Serum and liver were collected from dams on GD20 and PND22, and from offspring on GD20, PND4, PND14, and PND21. Serum T4, T3, and thyroid stimulating hormone (TSH) concentrations were measured by radioimmunoassay. Ethoxy-O-deethylase (EROD), pentoxyresorufin-O-deethylase (PROD) and uridine diphosphate glucuronyltransferase (UGT) enzyme activities were measured in liver microsomes. Custom low density Taqman qPCR arrays were used to measure hepatic mRNA expression of select cytochrome P450s, UGTs, sulfotransferases, transporters, and thyroid-hormone responsive genes at all developmental time points. Parent and conjugated TCS were quantified by LC/MS-MS in serum and whole liver homogenate at all developmental time points. Serum T4 decreased 30% in GD20 dams and fetuses, PND4 pups and PND22 dams at 300 mg/kg/day. Hepatic PROD activity increased 3-fold in PND22 dams and PND4 pups, and UGT activity was 1.5-fold higher in PND22 dams at 300 mg/kg/day. Only minor gene expression changes were found with TCS exposure, centered primarily on up-regulated expression of *Cyp2b* and *Cyp3a* isoforms in dams. Reductions of 30% in T4 for dams and GD20 and PND4 offspring with concomitant increases in PROD and UGT activity suggest

TCS may reduce T4 during development by a similar MOA to weanlings. Increased expression of *Cyp2b2* and *Cyp3a* isoforms in dams is consistent with our hypothesis that TCS activates CAR and/or PXR to perhaps transcriptionally up-regulate glucuronidation or sulfation of thyroid hormones *in vivo*. Analytical measurements of TCS in sera and liver demonstrated that transplacental exposure occurs, and that it is a more important route of exposure that lactation in rats, with PND14 and PND21 rats demonstrating lower internal exposures. This decreased exposure is consistent with the lack of any thyroid hormone changes at these ages. Overall the data suggest that up-regulated hepatic catabolism, particularly in dams, contributes to the observed maternal and early neonatal hypothyroxinemia.

2. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxyphenylether) decreases thyroid hormones (THs) in rats (Crofton *et al.*, 2007; Paul *et al.*, 2010a; Paul *et al.*, 2010b; Zorrilla *et al.*, 2009). This effect was hypothesized to result from the interaction of TCS with xenobiotic nuclear receptors, resulting in up-regulation of hepatic catabolism of THs and subsequent increased biliary excretion (Paul et al., 2010b). Previous work demonstrated modest maternal and early neonatal thyroxine (T4) decreases following oral administration to dams (Paul et al., 2010b), which is concerning because decreased maternal TH results in neurological deficits in rats (Gilbert *et al.*, 2000; Goldey *et al.*, 1998; Goldey *et al.*, 1995), as well as irreversible decreases in neurodevelopment and motor function in human children (Haddow *et al.*, 1999; Pop *et al.*, 2003; Pop *et al.*, 1999; Vermiglio *et al.*, 2004). Therefore it is important to determine whether TCS alters fetal THs, and characterize the mode-of-action (MOA) for

these TH decreases in rats in order to assess the potential for TCS-induced hypothyroxinemia in humans.

Maternal T4 concentration serves as a qualitative predictor of the neurological outcomes for offspring in rats and humans. The general physiological mechanism for this relationship begins with decreased maternal T4, resulting in decrease in the availability of free T4 to the fetus, leading to a decrease in the fetal brain concentrations of T3, which is necessary to activate thyroid receptors (TRs) for transcriptional regulation of the expression of genes involved in neurodevelopmental processes including myelination, neuronal and glial cell differentiation and migration during and pregnancy and the early postnatal period (Bernal, 2007; Williams, 2008; Zoeller *et al.*, 2004; Zoeller *et al.*, 2007). Supporting evidence for this observation comes from decades of epidemiological data that demonstrate a relationship between maternal hypothyroxinemia during the first trimester and early second trimester, typically defined as T4 below the 5-10 percentile, and decreased performance on psychomotor tests that evaluate mental ability, gross and fine motor skill coordination, and socialization of their children at 3 weeks (Kooistra *et al.*, 2006), 10-30 months (Berbel *et al.*, 2009; Henrichs *et al.*, 2010; Li *et al.*, 2010; Pop et al., 1999) and at 1-2 years (Pop et al., 2003). Maternal hypothyroidism and concomitant hypothyroxinemia during the early second trimester also corresponded to 4-10 point IQ deficits in children at 8 years of age (Haddow et al., 1999). Taken together these data support the critical role of maternal T4 during human development, and that the impacts of early gestational maternal hypothyroxinemia are lasting. Studies with rats also demonstrate a relationship between maternal hypothyroxinemia and altered neurodevelopmental outcomes in offspring, including changes in the cellular composition of several brain regions, expression of critical genes, and aberrant

neurobehavior. Maternal hypothyroidism and hypothyroxinemia change the cellular structure and maturation of the cortex and hippocampus (Berbel *et al.*, 2010; Gilbert, 2004; Lavado-Autric *et al.*, 2003; Sharlin *et al.*, 2008). Maternal hypothyroxinemia during gestation, in the absence of hypothyroidism or thyroid hormone effects in offspring, altered cortical neuron migration in white matter tracts (Lavado-Autric et al., 2003). Although fetal T4 production is active during the final week of gestation, maternal T4 supplements the fetal T4 supply to prevent altered cell migration in the cortex and hippocampus and decreased learning capacity (Berbel et al., 2010). Gene expression changes in the cortex related to cellular development and synaptic function, and in the hippocampus related to myelination and calcium signaling, were altered with 1 ppm propylthiouracil administered perinatally to rats, which resulted in maternal T4 and neonatal (PND14) T4 deficits of 32% and 51%, respectively, with no T3 or TSH effects (Royland *et al.*, 2008). These examples demonstrate that maternal, fetal, and neonatal hypothyroxinemia disrupt neurodevelopment, though the degree of hypothyroxinemia necessary to elicit adverse neurodevelopmental effects has not been well characterized.

Measurements of TCS in serum (Allmyr *et al.*, 2008; Allmyr *et al.*, 2006b; Allmyr *et al.*, 2009; Hovander *et al.*, 2002), breast milk (Adolfsson-Erici *et al.*, 2002; Allmyr *et al.*, 2006a; Dayan, 2007), and TCS metabolites in urine (Calafat et al., 2008; Wolff et al., 2007) demonstrate that humans are exposed to TCS, and highlight the potential for developmental exposure via maternal TCS exposures. A study of nursing mothers revealed that TCS concentrations in the serum of these mothers was 0.010 ng/g to 38 ng/g fresh weight (Allmyr et al., 2006a); of particular interest is that the TCS partitioned into serum at a much greater rate than into breast milk of these volunteers, suggesting that the oral dose to infants would

be more limited than the internal serum concentrations of the mothers (Allmyr et al., 2006a). For these mothers, TCS was detected in the women who had been exposed to personal care products containing TCS at much higher concentrations than mothers who had not been exposed to these types of products (Allmyr et al., 2006a). However, since TCS was present in all mothers regardless of intentional personal care product exposure, this indicates that multiple sources of exposure contribute to the total TCS exposure of the human population. The range of TCS detected in the breast milk of the mothers in this study, both control and exposed, was less than 0.018 ng/g-lipid to 0.95 ng/g-lipid (Allmyr et al., 2006a). In another study of five breast milk samples from Sweden, TCS concentrations ranged from less than 20 µg/kg-lipid to 300 µg/kg-lipid (Adolfsson-Erici et al., 2002). A study of 62 breast milk samples from milk donation banks in California and Texas demonstrated a range of TCS concentrations from below the limit of detection to 2100 µg/kg-lipid, with the majority of samples containing 200 µg/kg-lipid or less (Dayan, 2007). Based on these findings, and the standard volume of milk consumed by infants (Butte *et al.*, 1984; Butte *et al.*, 2002; EPA, 1997), Dayan used a conservative, high estimate of breast milk TCS concentration (1742 µg/kg-lipid) to estimate the maximum infant exposure to TCS as approximately 7.4 µg/kg/d. These findings underscore the potential for human developmental exposure via breast milk, when the potential impact of TH disruption may be most deleterious.

Previous work demonstrated that perinatal TCS exposure resulted in maternal and early neonatal hypothyroxinemia (Paul et al., 2010a); while the mechanism(s) responsible for the observed hypothyroxinemia during development have not been determined, we hypothesized that the MOA would be similar to that found in weanling rats exposed to TCS (Paul et al., 2010b). In a short-term exposure model with young adult rats, TCS decreased T4

and up-regulated markers hepatic catabolism, including uridine diphosphate glucuronyltransferase (UGT) activity, and *Ugt1a1* and *Sult1c1* expression (Paul et al., 2010b), in addition to increased *Cyp2b* and *Cyp3a1* expression, suggestive of hepatic nuclear receptor-mediated pathway. The main hypothesis of this work is that TCS decreases T4 in dams and offspring via up-regulation of hepatic catabolism, a demonstrated MOA for chemically-induced maternal and neonatal hypothyroxinemia (Zhou *et al.*, 2002; Zoeller *et al.*, 2005). This work also tests the hypothesis that transplacental exposure supplies a higher dose to fetuses than lactation exposure provides to neonates, suggesting that the observed recovery of T4 to control values in PND14 and PND21 offspring (Paul et al., 2010a) stems from toxicokinetic rather than toxicodynamic differences between progeny exposed through development.

3. Methods

Animals. Time-pregnant Long-Evans female rats (n=155), approximately 80-90 days of age, were obtained from Charles River Laboratories Inc. (Raleigh, NC) on gestation day (GD) 1 (defined as the day after vaginal plugs were observed), and were allowed five days of acclimation in an American Association for Accreditation of Laboratory Animal Care International (AALAC) approved animal facility prior to initiation of treatment on GD6. Animals were housed individually in plastic hanging cages (45 cm x 24 cm x 20 cm), with heat sterilized pine shavings bedding (Northeastern Products Corp., Warrenton, NC). Colony rooms were maintained at 21 ± 2°C with 50 ± 10 % humidity on a photo-period of 12L:12D. Food (Purina Rodent Chow #5001, Barnes Supply Co., Durham, NC) and water were provided *ad libitum*. Tap water (Durham, NC water) was filtered through sand, then

activated charcoal, and finally re-chlorinated to 4-5 ppm Cl^- before use in the animal facility. All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the US EPA.

Dams were orally exposed via gavage each day between 0800 and 1000 hr, with the exception of GD21, animals chemical was not administered to animals if there were signs of parturition. Figure 4.1 illustrates the dosing and tissue collection schedule. On GD21, dams were checked for the number of pups delivered at 0800, 1000, 1200, and 1500 hrs, and pups were aged as postnatal day (PND) 0 on the date of birth. All pups born from within a 24 hr period were considered to be the same age. On PND4, 14, and 21, offspring were counted, sexed, and group-weighed by sex. Average pup weight by sex was calculated by dividing the group weight by the number of pups. On PND4, litters were culled to 8 pups per litter, with the exception that litters comprised of less than 8 pups were not culled. Eye opening, determined as at least one eye open, was monitored once daily from PND11-17.

The current work included some serum and liver samples from previous work (Paul et al., 2010a) that reported only reproductive toxicity parameters, body weights, and serum T4 for the postnatal period. Two experimental blocks were completed with postnatal tissue collection from pups and dams (Table 4.1). Total combined sample numbers for T4 and hepatic microsomal assays were: 21, 12, 22, 22, 18 for 0, 10, 30, 100, and 300 mg/kg/day treatment groups. Due to limited serum volume particulary for early neonatal time points, TSH was measured in samples from Block 1 only (n=10 for 0, 30, 100 mg/kg/day and n=8 for 300 mg/kg/day), and T3 was measured in samples from Block 2 only (n=11 for vehicle control, n=12 for 10, 30, and 100 mg/kg/day, and n=10 for 300 mg/kg/day). For qPCR

experiments, n=6/treatment group using liver tissue from experimental Block 2. For analytical determination of TCS in sera and liver tissue samples, n=6/treatment group from experimental Block 2. From experimental Blocks 1 and 2, two dams including one vehicle control and one in the 300 mg/kg/day treatment group, were never pregnant; one dam in the 300 mg/kg/day group failed to deliver live pups; one other dam in the 300 mg/kg/day group died of unknown causes; and another was sacrificed early and excluded due to degenerative changes in one kidney and excessive urination.

An additional experimental block, Block 3 (n=55, with 11 animals/treatment group), was performed to collect fetal and dam tissues at GD20. Dams were dosed GD6 through GD19 only. The GD20 sample size for all measurements is as follows: n=11 for 0, 10, 30, and 100 mg/kg/day treatment groups, and n=10 for the 300 mg/kg/day treatment group, except for analytical determinations of TCS in sera and liver tissue samples, which used n=6/treatment group (Table 4.1). One dam in the 300 mg/kg/day group was hyperventilating and hyperactive, and the clinical veterinarian opinion was that the animal may have suffered abdominal torsion; this dam was sacrificed early on GD15.

Chemicals and treatment. Triclosan (5-chloro-2-(2,4-dichloro-phenoxy)phenol (CAS#3380-34-5; 98+ % pure) was obtained from Sigma-Aldrich Chemical Company (St Louis, MO, LOT#06415CD, Cat#524190-10G) and Ciba Grenzach GmbH (Germany, Lot#60023CL7). Mass spectrometry analysis revealed that the triclosan used was greater than 98.2% pure; the sample also contained 0.05% iso-triclosan, 0.12% 2,8-dichlorodibenzodioxin, and 0.1% 2,4,8-trichlorodibenzodioxin, but was free of biologically active dioxin compounds. The dosing solutions (0, 30, 100, and 300 mg/mL) were prepared

in corn oil (Sigma, Lot#117K0127), sonicated for 30 minutes, and stored in amber vials at room temperature. Solutions were prepared every 5-7 days. The 300 mg/kg/day dose partially precipitated within 24 hours and was therefore sonicated daily before use. All doses were mixed on a stir plate during the dosing period each morning. Dams were semi-randomly assigned to treatment groups by counter-balancing body weights. Administered volume was 1.0 ml corn oil/kg body weight; daily body weights were recorded, and administered volumes were adjusted daily by weight. Prior to the sacrifice of dams and fetuses on GD20, pups on PND4 (only culled pups), 14 and 21 (one pup per sex per litter), animals were moved to a holding room, dams were weighed and dosed, pups were weighed, and all animals were acclimated for a minimum of 30 min. Tissue collection was conducted between 0800 and 1200 hrs in an adjacent room with a separate air supply. The time of necropsy within the 4-hr period was balanced among dose groups to control for time-of day effects on thyroxine levels (Dohler *et al.*, 1979; Jordan *et al.*, 1980). Trunk blood was collected from one male and one female pup per litter and pooled into one tube. Blood from dams was collected after decapitation on PND22, 24 hr after the final dose. Blood was collected into serum separator tubes (Beckton Dickinson, 36-6154). Serum was obtained after clotting whole blood for 30 min on ice, followed by centrifugation at 1278x g at 4°C for 30 min. Serum samples were stored at -80°C until analysis. Liver was obtained from all offspring and dams, divided into sections, and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Thyroid hormone assays. Serum total T4 and total T3 was measured in duplicate by standard solid-phase Coat-A-Count radioimmunoassay (RIA) kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA). T3 was not measured for GD20 fetuses nor PND4 neonates due to limited serum volumes and a lack of effect on any other time point. Serum

TSH concentrations were analyzed in duplicates with a double antibody RIA method (Greenwood *et al.*, 1963) with some modification (Zorrilla et al., 2009). The TSH radioimmunoassays were performed using materials supplied by the National Hormone and Pituitary Agency: iodination preparation (I-9-TSH); reference preparation (RP-3); and antisera (S-6-TSH). Iodination material was radiolabeled with ^{125}I (Perkin Elmer, Shelton, CT). Assay variation was assessed using the multivalent control module (Siemens Medical Solutions Diagnostics, Los Angeles, CA; Lot021) to measure low, medium, and high total T4, T3, and TSH values before and after measuring the experimental samples. Intra-assay coefficients of variance for all assays ranged were below 15%, and the inter-assay coefficients of variance for T4, T3, and TSH were 5.8, 10.8, and 13.1% for assays conducted over a two year period. Total serum T4 and TSH were calculated as ng T4/ml serum, and total serum T3 was calculated as ng T3/dL serum.

Total serum T4 for GD20 fetuses was measured in duplicate by a different radioimmunoassay method as described elsewhere (Bansal *et al.*, 2005; Gauger *et al.*, 2004). Briefly, each assay contained: 5 µl of rat serum, 100 µl barbital buffer (0.11 M barbital pH 8.6, 0.1% w/v 8-anilino-1-napthalene-sulfonic acid ammonium salt (ANS), 15% bovine γ-globulin Cohn fraction II, 0.1% gelatin), 100 µl anti-T4 (rabbit, Sigma) diluted to provide a final concentration of 1:21,000, and 100 µl ^{125}I-T4 (diluted to yield a total of 12,000–15,000 cpm; Perkin Elmer/NEN). Triplicate standards ranging from 4 ng/mL to 256 ng/mL were prepared from T4 (Sigma). Following a 30 min incubation at 37°C, the tubes were chilled on wet ice for 30 min. Antibody-bound radiolabeled T4 was precipitated by addition of 300 µl ice-cold polyethylene glycol 8000 (20% w/w; Sigma). Tubes were then centrifuged at 1800 ×

g for 20 min at 4 °C, and the supernatant was aspirated and counted on a gamma counter (Packard CobraII). The lower limit of detection for this method was 2.0 ng/mL.

Microsome preparation and EROD and PROD assays. Liver microsomal fractions were prepared as described previously (DeVito *et al.*, 1993) and standardized using total protein (Bio-Rad, Richmond, CA). Hepatic microsomal EROD and PROD activities were assayed using a fluorometric microplate reader (Spectramax Gemini XPS, Molecular Devices/MDS Analytical Technologies,Toronto, Canada). Each well in polystyrene 96-well plates (Nunc ThermoFisherScientific, Rochester, NY) contained a total of 235 ul, including 50 ul of substrate (1.5 nM ethoxyresorufin or pentoxyresorufin), 50 µL of diluted microsomes, and 110 µL of 0.05 mM Tris buffer (pH 8.0). Microsomes were diluted 1:10 for samples and 1:100 for the positive control in order to maintain data points within the linear region of the standard curve. An aliquot (25 µL) of NADPH was added to initiate the reaction. The fluorescence signal was measured every 33 seconds for five min at 37ºC after reaction initiation. The rate of resorufin formation was estimated by calculating Vmax/min, using a resorufin standard curve to extrapolate resorufin concentrations in the reaction. A similar method was used to measure hepatic microsomal PROD activity, using pentoxyresorufin as a substrate. Both EROD and PROD values were calculated as picomoles (pmol) resorufin per milligram protein per minute. Two positive controls were used to facilitate inter-assay and mechanistic comparison: pooled microsomes from rats acutely exposed to 10 µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 300 mg/kg Aroclor, and pooled microsomes from rats that received 4-day intraperitoneal exposure to phenobarbital (81 mg/kg/d) or PCN (50 mg/kg).

UGT activity assay. UGT activity for T4 was measured by the method of (Beetstra *et al.*, 1991) as modified by (Zhou *et al.*, 2001). Detergent such as Brijj 56 was not included due to

the potential for increased basal T4 glucuronidation (Craft *et al.*, 2002). Two positive controls were used to facilitate inter-assay and mechanistic comparison: pooled microsomes from rats acutely exposed to 10 µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 300 mg/kg Aroclor, and pooled microsomes from rats that received 4-day intraperitoneal exposure to phenobarbital (81 mg/kg/d) or PCN (50 mg/kg).

mRNA preparation and analysis. For each of the six time points included in this study, the n=6/treatment group, with all treatment groups represented (0, 10, 30, 100, 300 mg/kg/day). Extraction of mRNA from frozen tissue was performed using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Approximately 60 mg of frozen tissue was homogenized and split between two Qiagen QIAShredder columns to assure complete homogenization. RNA extraction was processed in duplicate for each sample per the directions. Contaminating DNA was removed on the Qiagen Mini-Kit columns using the Qiagen DNase kit. RNA content and purity was assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.; Wilmington, DE) at absorbances of 230, 260, and 280 nm for the duplicate RNA samples. Samples used in experiments were selected from duplicates based on optimizing the 260/230 and 260/280 ratios to 2.0 (range was 1.80 – 2.14 for 260/230 and 1.95 – 2.11 for 260/280). The average 260/230 ratio for samples used was 2.02, and the average 260/280 ratio for samples used was 2.06.

RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA), as per the manufacturer directions. Two micrograms of RNA were added to each 20 µL reverse transcription reaction.

qPCR gene expression assays were performed using Custom Taqman ® Low Density Arrays (Applied Biosystems, Foster City, CA; Cat.#: 4309169), which are 384-well microfluidic cards preloaded with 23 unique gene targets, including several potential endogenous controls, each performed in duplicate for each sample. Eight samples were loaded per card, in a semi-random order, where samples for each developmental time point were randomized and then run as separate cohorts by time point. Following sample loading, plates were centrifuged at 1200 rpm, 24ºC, twice for 1 min before being run on a ABI Prizm 7900HT (Applied Biosystems) .

qPCR data sets were analyzed using a relative quantification method ($2^{-\Delta\Delta C}{}_{T}$) to describe the change in expression of the target experimental gene relative to an endogenous reference gene (Livak *et al.*, 2001). Choice of an endogenous reference gene was based on constant gene expression across all of the dosing groups for each developmental time point (Dunn *et al.*, 1998; Livak et al., 2001). The candidate endogenous reference genes Actb, GAPDH, Rps18, and Rpl13a were run for each sample (Pohjanvirta *et al.*, 2006). Since there can be differential expression of common endogenous control genes at different developmental ages, choice of the endogenous control gene was performed by picking the gene for which the standard deviation from the mean of treatment group expression values was the smallest, as per the *a priori* criteria. Rps18 was chosen for GD20 and PND22 dams, GD20 fetuses, and PND21 offspring. Actb was selected for PND4 and PND14 offspring. However, using Rps18 for all time points did not significantly alter the results (data not shown). Choice of the genes included on the low density array was contingent upon three criteria: involvement of the isoform in TH metabolism and transport in rats, regulation of the isoform by nuclear receptors AhR, CAR, PXR, and/or PPAR, and sensitivity to tissue levels

of thyroid hormones. A list of the genes and Taqman identifiers for these is listed in Table 4.2.

Analytical measurement of TCS in sera and liver. Serum was obtained from animals iver homogenate were made by homogenizing 0.5 g of tissue in 2 mL of diH_2O. TCS content in these tissues was analyzed by liquid chromatography-mass spectrometry (LC-MS) utilizing isotope dilution (Agilent 1200 high performance liquid chromatography [HPLC] with API 4000 Triple Quadrapole MS), similar to the method previously reported for measuring parent TCS (Fort *et al.*, 2010). The mass spectrometry turbolon spray was utilized in negative mode for the negative TCS ion, with multiple reaction monitoring detection for parent ion (286.0 amu) and product ion (35.1 amu). The calibrated range for TCS determination was 1-1000 ng/mL with $^{13}C_6$-TCS as an internal standard in blank serum. Serum and liver homogenate were analyzed undiluted unless preliminary data suggested out-of-range values for TCS.

Parent TCS was measured in samples that contained 100 µL serum or liver homogenate and a 10 ng $^{13}C_6$-TCS spike. Acetonitrile (200 µL) was added to precipitate protein. Samples were vortexed (1 min), briefly centrifuged, and then analyzed by LC/MS. The limit of detection for parent TCS was 1 ng/mL. Conjugated and parent TCS, or total TCS, were measured following an enzymatic hydrolysis step with combined glucuronidase and sulfatase and precipitation with acetonitrile. Serum or liver homogenate (100 µL) was spiked with 10 ng 13C-TCS and a glucuronide/sulfate standard tracer. After addition of 50 µL of β-glucuronidase and sulfatase enzymes (0.2 mg of enzyme total per sample), a 4 hr incubation at 37ºC was performed. Acetonitrile (400 µL) was added to stop the reaction and precipitate the protein. Samples were vortexed (1 min) and centrifuged. The supernatant

containing total TCS was then analyzed by LC/MS. The limit of detection for total TCS was 5 ng/mL serum or liver homogenate.

Data Analysis. Dam bodyweight data for prenatal and postnatal stages were analyzed separately using repeated measures ANOVAs (SAS 9.1, SAS Institute, Cary, NC), followed by mean contrast testing with Duncan's New Multiple Range Test ($p<0.05$), with dose and animal as independent variables. All other data were analyzed by ANOVAs, with significant main effects followed by Duncan's New Multiple Range Test. Gestation length was defined as the period from GD0 to the day of birth. The viability index was calculated as the number of pups alive on PND0 divided by the number alive on PND4 per litter prior to culling. Sex ratio was calculated as the number of female pups divided by the number of male pups. Eye opening was calculated as mean percent of pups with at least one eye open in a litter for each treatment.

Benchmark dose (BMD) and lower-bound confidence limit (BMDL) estimates were determined using USEPA Benchmark Dose Software (BMDS Version 2.0beta) as previously described (Crofton et al., 2007; Zhou et al., 2001; Zhou et al., 2002; Zorrilla et al., 2009). The benchmark response (BMR) (EPA, 2000) was set at a 20% decrease in thyroxine, reflecting previous use of this BMR in the literature Crofton and Zhou refs. The BMD was calculated from a model fit to the data. The BMDL (lower-bound confidence limit) was calculated as the 95% lower confidence interval.

4. Results

Gestational maternal exposure to TCS did not affect the number of fetuses [$F(4,48)=1.13$, $p<0.3549$], and there were no effects of treatment on maintenance of pregnancy, as indicated by the lack of treatment-related difference in the number of implantation sites and fetuses on

GD20 [$F(4,48)=1.41$, $p<0.2452$]. Perinatal maternal triclosan exposure did not affect any reproductive parameters including: gestation length [$F(3, 93)=0.05$, $p<0.8155$], litter size [$F(3,93)=0.62$, $p<0.4314$], viability index [$F(3,92)=3.55$, $p=0.0627$], or sex ratio [$F(3,92)=2.93$, $p<0.0901$]. Treatment did not elicit effects on the day of eye opening [$F(1,90)=1.51$, $p<0.2216$] (data not shown). Further, no gross terrata were observed in any of the pups, and offspring viability was unaffected by treatment.

No treatment-related clinical signs of toxicity were observed in the dams or offspring during the either the 19 or 36 day course of TCS treatment. There was no main effect of treatment on dam body weight during gestation [$F(4,91)=1.65$, $p<0.1681$]. However, dam body weights for the 300 mg/kg/day treatment group decreased by approximately 10% throughout the postnatal period ($p<0.05$). This observation is supported by a main effect of treatment on the body weight of dams [$F(4,83)=4.80$, $p<0.0016$], though there was no postnatal-day and treatment interaction [$F(80,1660)=1.76$, $p<0.7005$]. There were no effects of treatment on pup body weight, male or female, at ages PND4, PND14, or PND21 (data not shown). There were no effects of treatment on dam liver weight [$F(4,89)=1.31$, $p<0.2740$] or liver-body weight ratio [$F(4,85)=1.52$, $p<0.2025$].

A main effect of TCS treatment on serum total T4 was observed, with a significant decrease of 15% for PND22 dams with 100 mg/kg/day of TCS [$F(4,85)=12.77$, $p<0.0001$], and 30% for both the GD20 dams [$F(4,48)=3.78$, $p<0.0095$] and the PND22 dams with 300 mg/kg/day of treatment (Figure 4.2). The no-observed-effect level (NOEL) for TCS and T4 was 100 mg/kg/day for GD20 dams and 30 mg/kg/day for PND22 dams. The BMDs for serum T4 concentrations were 124 and 115 mg/kg/day with 95% lower confidence limits of 25.2 and 62.1 mg/kg/day for GD20 and PND22 dams, respectively (Table 4.3). For

offspring, there was a main effect of TCS treatment on serum total T4, except that this effect was limited to GD20 fetuses and PND4 neonates. Serum total T4 was decreased by 25% and 29% in fetuses from the 100 and 300 mg/kg/day treatment group [$F(4,48)=2.91$, $p<0.0312$] and by 26% in PND4 pups from the 300 mg/kg/day treatment group [$F(4,85)=5.02$, $p<0.0011$]. The computed BMDs for serum T4 were 95.4 and 150 mg/kg/day with 95% lower confidence limits of 33.0 and 61.8 mg/kg/day for GD20 fetuses and PND4 neonates, respectively (Table 4.3). There were no effects of treatment on serum T4 for PND14 [$F(4,89)=1.44$, $p<0.2261$] or PND21 [$F(4,89)=1.59$, $p<0.1831$] neonates from any treatment group.

There were no effects of treatment on serum T3 on any dam or offspring timepoint (data not shown; see Appendix 4). Only sera from GD20 dams [$F(4,49)=0.82$, $p<0.5215$], PND22 dams [$F(4,51)=2.23$, $p<0.0783$], PND21 pups [$F(4,51)=0.37$, $p<0.8296$], and PND14 pups [$F(4,52)=0.52$, $p<0.7238$] were analyzed. Offspring from GD20 and PND4 were not tested due to limited serum volumes. There were no effects on T3 for the GD20 or PND22 dams, both of which demonstrated T4 reductions.

There were no effects of treatment on serum TSH on any age tested, including GD20 dams [$F(4,49)=0.64$, $p<0.6382$], PND22 dams[$F(3,34)=0.27$, $p<0.8478$], PND21 pups [$F(3,34)=0.42$, $p<0.7406$], PND14 pups [$F(3,33)=0.52$, $p<0.6733$], or PND4 pups [$F(3,27)=2.01$, $p<0.1363$] from any treatment group (Figure 4.3). Serum obtained from GD20 fetuses was not tested due to limited serum volumes and a lack of effect on TSH for GD20 dams, PND22 dams, and PND4 neonates, which did demonstrate effects on T4.

TCS increased PROD activity in an age-dependent manner that mostly reflected the ages associated with T4 decreases in this study. Increased hepatic microsomal PROD

activity was observed for PND4 neonates and PND22 dams only (Figure 4.4). PROD activity increased to 220 ± 38 percent of vehicle control for PND4 pups [$F(4,51)=8.19$, $p<0.0001$] and to 309 ± 44 percent of vehicle control for PND22 dams [$F(4,85)=9.55$, $p<0.0001$]. No significant effects were observed in GD20 dams [$F(4,48)=1.89$, $p<0.1272$] despite an effect of TCS on T4 for animals at this time point. No effects of TCS were observed on PROD activity induction for PND14 [$F(4,85)=2.34$, $p<0.0616$) or PND21 [$F(4,85)=1.64$, $p<0.1705$] neonates, and GD20 fetuses were not tested, as Cyp2b activity surges at parturition in rats (Borlakoglu *et al.*, 1993), and we were unable to measure any activity using our assay. TCS decreased EROD activity for GD20 dams [$F(4,48)=9.04$, $p<0.0001$] , PND22 dams [$F(4,85)=20.03$, $p<0.0001$], and PND14 neonates [$F(4,85)=11.82$, $p<0.0001$] by approximately 40-50% for the 100 and 300 mg/kg/day treatment groups (Figure 4.4). No effects of TCS were seen on EROD activity for PND4 [$F(4,51)=0.44$, $p<0.7797$] or PND21 [$F(4,85)=1.34$, $p<0.2616$] neonates, and GD20 fetuses were not tested.

Hepatic microsomal UGT-T4 activity was induced by TCS only for PND22 dams [$F(4,84)=3.00$, $p<0.0228$] to approximately 150% of vehicle control with no effect of block (Figure 4.5). UGT-T4 activity for GD20 dams [$F(4,48)=0.57$, $p<0.6849$] and PND4 neonates [$F(4,49)=0.70$, $p<0.5954$] was not affected by treatment, and UGT-T4 activity was not measured for ages which did not have a treatment-related effect on T4 (PND14, PND21 neonates) or GD20 fetuses, due to limited tissue. Positive controls representing prototypical microsomal enzyme inducers have been included for comparison. Positive control 1 is a pool of microsomes obtained from animals treated once by gavage with either TCDD (10 µg/kg) or Aroclor 1254 (300 mg/kg); this pool induced UGT activity 202 ± 19 percent of control. Positive control 2 is a pool of microsomes obtained from animals treated for four days by

intraperitoneal injection with either phenobarbital (81 mg/kg/d) or PCN (50 mg/kg); this pool failed to induce UGT-T4 activity in this assay (113 ± 7.6 percent of control).

TCS treatment had minor effects on CYP gene expression, producing few and only modest significant effects at any time point included in this study (Figure 4.6). *Cyp2b2* expression was increased to 2.8141 ± 0.5223 and 2.4767 ± 0.4061 fold-control at 300 mg/kg/d for GD20 dams [$F(4,25)= 4.12$, $p<0.0107$] and PND22 dams[$F(4,25)=4.35$, $p<0.0083$], respectively. *Cyp3a1* was induced 2.0390 ± 0.2745 fold-control for PND22 dams only at 300 mg/kg/day [$F(4,25)=3.55$, $p<0.0200$], and there was also a strong increasing trend for *Cyp3a2* in PND22 dams as well (2.6 fold-control at 300 mg/kg/day, $p<0.0529$) (data not shown). *Cyp3a9* expression was induced to 1.8286 ± 0.1378 fold-control for GD20 dams [$F(4,25)=4.17$, $p<0.0100$], and values for *Cyp3a9* expression in PND14 and PND21 pups could not be reported due to low expression. *Cyp4a2* was induced in GD20 fetuses to 1.8398 ± 0.2044 fold-control [$F(4,25)=4.46$, $p<0.0074$]. Any TCS effects on hepatic CYP expression appear to be isoform- and age-dependent; no single CYP was uniformly changed by TCS treatment in all age groups. With no effects of TCS on T4 concentrations at PND14 and PND21, little to no effect on hepatic gene expression was anticipated. Overall the data suggest that TCS up-regulated expression of *Cyp2b* in adult dams, with some increased *Cyp3a* expression, though for different isoforms in the pregnant versus the nursing dam.

There were no statistically significant treatment related effects on hepatic gene expression of *Ugt1a1* or *Ugt1a6* for any of the developmental time points included in this study (Figure 4.7). Although GD20 fetuses appear to demonstrate increased *Ugt1a1* expression (1.88 ± 0.56), variability in the data prevents a statistically significant effect at

300 mg/kg/day [$F(4,25)=0.95$, $p<0.4537$]. Hepatic sulfotransferase expression of *Sult1b1* was not affected by treatment for any of the time points included. However, expression of *Sult1c3* was induced for PND14 and PND21 neonates to 1.98 ± 0.23 fold-control [$F(4,25)=2.86$, $p<0.0444$] and 2.44 ± 0.32 fold-control [$F(4,25=4.81$, $p<0.0051$], respectively. There were no significant changes in gene expression in GD20 dams, PND22 dams, GD20 fetuses, or PND4 neonates, the groups which experienced decreased serum T4 at 300 mg/kg/day.

There were no significant dose-dependent effects of TCS treatment on transporter gene expression for the isoforms chosen: *Mct8*, *Mrp2*, and *Oatp1a4* (data not shown; refer to Figure 4.10). *Oatp1a4* basal expression was too low in GD20 fetuses to be included in our relative quantitative analysis, and so values for GD20 fetuses are were not calculated. No effects on *Mct8* expression were noted for any of the positive controls either . All of the prototypical inducers appeared to increase *Mrp2* expression (Figure 4.9); *Mrp2* was induced to 1.90 ± 0.41, 2.54 ± 0.11, 3.57 ± 0.39, 2.06 ± 0.06, and 2.11 ± 0.21 fold-control by TCDD, Aroclor 1254, PCN, PB, and pooled TCDD and Aroclor 1254, indicating that the strongest induction was by the prototypical PXR agonist, PCN. Only PCN capable of noticeable *Oatp1a4* induction (4.82 ± 0.34 fold-control).

TCS did not affect the gene expression of TH-responsive genes, which we defined as *DioI, Me1,* and *Thsrp* (also known as *Spot14*) at any time point included in this study (Figure 4.8). Though *Thrsp* expression appears increased for PND14 pups at 300 mg/kg/day (3.65 ± 2.45 fold-control), there was extensive variability in the data set preventing a significant effect.

For comparison, the positive control set produced changes in gene expression for *Me1*: 11.94 ± 1.73, 14.80 ± 0.74, 5.47 ± 0.54, 6.59 ± 1.51, 12.73 ± 1.24 fold-control for TCDD, Aroclor 1254, PCN, PB, and a pool of TCDD and Aroclor 1254, respectively (Figure 4.9). Expression of *Thrsp* was also altered by treatment with these prototypical inducers, but the magnitudes of the changes were more modest; TCDD, Aroclor 1254, and pooled TCDD and Aroclor 1254 induced *Thrsp* expression to 1.90 ± 0.30, 1.66 ± 0.16, and 1.69 ± 0.22 fold-control, whereas PCN and PB seemed to have no effect (0.68 ± 0.17 and 1.03 ± 0.17 fold-control, respectively).

Clearly there were few effects of TCS exposure on hepatic gene expression of the target genes chosen; the main effects were on dam expression of *Cyp2b* and *Cyp3a* isoforms. A visualization of all of the data for the target genes and animal ages is provided in Figure 4.10.

Analytical measurement of total TCS in sera, defined here as the parent TCS and the glucuronide and sulfate conjugates, demonstrated that GD20 dams had the most total TCS in their serum, followed closely by GD20 fetuses and PND4 neonates (Figure 4.11). Total TCS appeared in serum per the following relationship: GD20 dams > GD20 fetuses > PND4 neonates > PND22 dams > PND14 neonates >> PND21 neonates. The concentration of parent TCS in sera suggested that TCS is predominantly found as conjugate in the systemic circulation of rats, with the exception of PND4 neonates, which had markedly increased parent TCS in their sera. This is further illustrated by examination of the percent parent (Figure 11C) in PND4 neonates, which is consistently measured as 30-40% of the total TCS found in their circulation. In contrast all of the other time points included appear to have approximately 0-10% parent TCS in their sera.

Measurement of total TCS in liver homogenate suggested a similar relationship to the sera data set; GD20 dams had the highest concentration of total TCS in their livers, followed closely by PND22 dams, GD20 fetuses, and PND4 neonates (Figure 4.11). Total TCS appeared in liver per the following relationship: GD20 dams > PND22 dams > GD20 fetuses > PND4 neonates > PND14 neonates >> PND21 neonates, indicating that as expected, the animals that were directly gavaged with TCS, i.e. the dams, had the highest target tissue dose of total TCS. The relative concentrations of total TCS in liver appear to have been consistent with the concentration of parent TCS in liver. In contrast to the concentrations of parent TCS in sera, parent TCS appears to have comprised a larger fraction of the TCS present in the livers of these animals. For PND21 neonates, which demonstrated the lowest target tissue doses, nearly all of the TCS was parent (80-100%), with the amount of conjugate present increasing with the amount of total TCS exposure. For GD20 fetuses, which demonstrated greater concentrations of total TCS exposure, approximately half of the TCS present in their livers appears to have been parent, except for the vehicle control group, which appeared to have about 20% parent TCS. However, the amounts of TCS present in the vehicle control group are close to zero. For all of the other time points, including GD20 dams, PND22 dams, PND4 neonates, and PND14 neonates, the amount of parent TCS in the liver was about 70-80%, except for the vehicle group, which again had trace amounts of TCS that were about 60% parent TCS.

Figure 4.12 illustrates the amount of total TCS in sera (A) and liver (B) at 300 mg/kg/day, the dose that elicited T4 effects in GD20 dams and fetuses, PND4 neonates, and PND22 dams. A decreasing internal exposure in offspring throughout lactation is evident, with an apparent relationship that is consistent for both sera and liver samples: GD20 fetuses

> PND4 neonates > PND14 neonates >> PND21 neonates. For total TCS in sera and liver, it appears that for TCS to induce equivalent hypothyroxinemia in GD20 and PND22 dams, a 30% reduction in T4, GD20 dams maintain a higher internal concentration of TCS. Similarly, GD20 fetuses and PND4 neonates also experienced an approximate 30% decrease in T4 in the 300 mg/kg/day treatment group, and their sera and liver concentrations appear to be nearly the same as the two groups of dams.

5. Discussion

This work confirms preliminary findings and provides the first report of several key results following developmental TCS exposure. First, dams, fetuses, and young neonates are susceptible to TCS-induced hypothyroxinemia, confirming previous observations (Paul et al., 2010a), with no effects on T3 or TSH concentrations at any time point. Serum total T4 concentrations were reduced in GD20 fetuses and PND4 neonates, but recovered to control values for PND14 and PND21 neonates, representing a unique pattern of T4 effects in offspring. Second, we observed modest increases in markers of hepatic microsomal enzyme induction that were consistent with moderate decreases in serum T4 and our previously hypothesized MOA. Finally, analysis of serum and liver TCS content revealed that PND14 and PND21 neonates had lower internal concentrations of TCS, which suggested that toxicokinetics may account for the recovery of serum T4 to control values in these animals. As a whole the data indicate that TCS is a low-potency and low-efficacy thyroid hormone disruptor; developmental exposures of up to 300 mg/kg/day resulted in moderate decreases in T4 and a minor up-regulation of hepatic catabolism, which supports our hypothesis that TCS may increase catabolism and elimination of T4, potentially contributing to the observed systemic T4 decreases.

Gestational and perinatal maternal exposure to TCS resulted in mild hypothyroxinemia in GD20 dams and PND22 dams, as well as in GD20 fetuses and PND4 neonates. The approximate 30% reductions in T4 observed for these four groups is consistent with previous studies in rats (Crofton et al., 2007; Paul et al., 2010a; Paul et al., 2010b). However, this is in contrast to one recent report, which demonstrated 30-40% decreases in maternal T4 with 50 mg/kg/d TCS administered via drinking water to Wistar rats from 8 days prior to mating through lactation (Rodriguez et al., 2010); this study also demonstrated serum T3 decreases of 15-20% at 50 mg/kg/d that were not observed in the present work with doses up to 300 mg/kg/d. The current work also confirms previous findings of no reproductive toxicity, with no effects of treatment on gestation length, viability, eye opening, sex ratio, or pup body weights, with minor decreases in dam bodyweight observed during the postnatal period for the 300 mg/kg/d group (Paul et al., 2010a).

The observed hypothyroxinemia in dams, fetuses, and young offspring demonstrate and confirm the previous findings of TCS-induced hypothyroxinemia following developmental and juvenile exposures (Crofton et al., 2007; Paul et al., 2010a; Paul et al., 2010b; Zorrilla et al., 2009). These data also confirmed the recent report of a unique pattern of effects on offspring during the postnatal period: a decrease in T4 at an early age (PND4) followed by a return to control T4 concentrations at PND14 and PND21 (Paul et al., 2010a). Oral exposure to the dams produced similar decreases of approximately 30% in the dams at GD20 and PND22, and GD20 fetuses and PND4. This is consistent with our previous observations of 30% decreases in serum total T4 following perinatal exposure (Paul et al., 2010a). An additional study, though not a developmental exposure, of the effects of TCS on serum T4 in Long-Evans weanling females exposed for 4 days to TCS also demonstrated 30% decreases

with 300 mg/kg/day TCS (Crofton et al., 2007; Paul et al., 2010b). However, different rat strains, gender, chemical source, and exposure duration may impact the relative potency and efficacy of TCS exposure and limit the ability to compare studies. Another non-developmental exposure study that employed a 31-day exposure with juvenile male Wistar rats demonstrated 50% decreases in serum total T4 with 30-100 mg/kg/day TCS (Zorrilla et al., 2009), suggesting greater potency in their model. Currently there is no evidence to inform a hypothesis about the differences in potency between the study in juvenile weanling male Wistar rats and studies in Long-Evans female rats, particularly pregnant rats, but a plausible theory might be that these animals demonstrate both toxicodynamic and toxicokinetic differences in response to TCS exposure.

The unique pattern of the T4 in offspring in response to maternal TCS exposure during the postnatal period was surprising and confirmed our previous preliminary findings (Paul et al., 2010a). The lack of effect on T4 concentrations in PND14 and PND21 offspring during the lactation period incited further questions: (1) does TCS elicit a different toxicodynamic response in perinatally-exposed PND14 and PND21 neonates with no subsequent effects on T4 concentrations; or (2) what is the relative dose received by all of the offspring, and do PND14 and PND21 neonates receive as much TCS as GD20 fetuses and PND4 neonates? This work tested the hypothesis that PND14 and PND21 neonates were not receiving the same level of exposure to TCS as GD20 fetuses and PND4 neonates. Previous studies on polybrominated diphenyl ethers, polychlorinated biphenyls, propylthiouracil, and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) have demonstrated that postnatal thyroid hormones are usually affected by perinatal (i.e., gestational and lactation) maternal xenobiotic exposure during the entirety of the postnatal lactation period (Crofton *et al.*, 2000; Gilbert *et al.*, 2006;

Goldey et al., 1995; Kodavanti *et al.*, 2010; Morse *et al.*, 1993; Royland et al., 2008; Sharlin *et al.*, 2006; Zhou et al., 2001; Zhou et al., 2002). Previous studies with rats exposed from 27-29 days of age demonstrated effects on T4 and increases in markers of hepatic catabolism (Paul et al., 2010b), and microsomal enzymes, including CYPs and UGTs, are known to be inducible in PND14 and PND21 neonates (Zhou et al., 2002), so differences in the ontogeny of hepatic enzymes seemed an unlikely explanation. Measured TCS content in the sera and liver of the dams demonstrate similar serum and liver concentrations of both the parent and total TCS at GD20 and PND22. However, TCS concentrations in fetal and neonatal serum and liver depend on the age of the animal. Parent and total TCS concentrations decreased in PND14 and PND21 offspring when compared to GD20 fetuses and PND4 pups (Figure 4.12), and suggest that a lack of effect on T4 at PND14 and PND21 are due to the much lower exposures PND14 and PND21. Internal serum and liver concentrations of TCS was measured as a surrogate for exposure in offspring, so the exact transplacental and oral lactation exposures are not described, and the impact of different absorption rates by developmental life stage is unknown. The GD20 fetuses demonstrated the highest concentration of total TCS in offspring, which was close to the internal concentration for GD20 and PND22 dams. The finding that nearly all of the TCS present in the GD20 fetus was TCS-conjugate is consistent with previous reports in sheep that demonstrated fetal capacity to glucuronidate and sulfate acetaminophen, but an inability to clear the conjugates or hydrolyze the conjugates back to parent (Wang *et al.*, 1986). The total TCS dose received via transplacental exposure appeared to be the greatest, but the magnitude of the effect on T4 concentrations in GD20 fetuses was still comparable to dams and PND4 neonates, indicating that GD20 fetuses may be less or similarly sensitive to T4 perturbation than other life stages

exposed in this study. The lowest internal serum concentration of TCS that elicited a statistically significant effect on T4 was found in PND22 dams, which experienced a 15% decrease in serum T4 with approximately 5120 ng/mL total TCS measured in their sera. Comparing this with the highest known reported human plasma concentration of total TCS, 303 ng/mL, reported by Allmyr et al. after conducting a 14-day exposure to TCS-containing toothpaste using a normal dental hygiene routin (Allmyr *et al.*, 2009), yields a ~17-fold difference in exposures. Importantly, the individual human exposure that produced a serum concentration of 303.4 ng/mL resulted in only a slight increase in a serum marker of CYP3A4 activity and no changes in serum free T4, with no statistically significant changes in CYP3A4 activity or THs for the study group as a whole (Allmyr et al., 2009). Based on a comparison of the data presented in this work and the work of Allmyr et al., at a minimum it can be ascertained that humans are not 17-times more susceptible to TCS-induced hypothyroxinemia than rats.

Indicators of Phase I metabolism were slightly increased in response to TCS exposures that produced mild hypothyroxinemia; PROD activity increases were observed in PND4 offspring and GD20 and PND22 dams, and *Cyp2b* and *Cyp3a* isoform-specific expression increases were observed in dams, consistent with activation of the constitutive androstane receptor or pregnane X receptor. Increased hepatic microsomal PROD activity was consistent with increased hepatic catabolism, though the observed effects were minor. TCS-induced increases in PROD activity, indicative of Cyp2b activity in the rat, were fairly consistent with observed reductions in T4, and were concordant with previously reported PROD activity and/or Cyp2b1/2 protein increases following *in vivo* non-developmental TCS exposures in rats (Hanioka *et al.*, 1997; Paul et al., 2010b; Zorrilla et al., 2009) and *in vitro*

TCS exposures in rat microsomes and hepatocytes (Hanioka *et al.*, 1996; Jinno *et al.*, 1997). PROD activity was increased 2-3-fold control for PND22 dams and PND4 neonates; GD20 dams also demonstrated T4 deficits, but no significant effects on PROD activity were observed. PROD activity was not measured in GD20 fetuses due to limited tissue and low CYP activity (de Zwart *et al.*, 2008). The lack of effect on PROD activity for GD20 dams may be due to their unique metabolic status during pregnancy; several CYPs including Cyp2b2 protein are known to be decreased in rat liver during pregnancy and return to pre-pregnancy levels during lactation (He *et al.*, 2005b; He *et al.*, 2005a). Further evidence suggests that Cyp2b1/2 may be less susceptible to protein induction or gene expression increases resulting from PB or PCN treatment during pregnancy, while other Phase I enzymes such as Cyp3a1 remain inducible (Ejiri *et al.*, 2005a; Ejiri *et al.*, 2005b). No increases in PROD activity were observed for PND14 or PND21 pups. However, these CYPs are functional at these ages, with assessment of the ontogeny of Cyp2b1/2 activity demonstrating high activity during the mid- to late-lactation period (de Zwart et al., 2008); further, PROD is inducible at these postnatal ages as demonstrated by perinatal exposure to DE-71 (Szabo *et al.*, 2009; Zhou et al., 2002). Based on analytical measurement of TCS in the sera and livers of these PND14 and PND21 offspring, we propose that the lack of effect on PROD activity is consistent with a reduced TCS exposure rather than a toxicodynamic difference between PND14 and PND21 offspring and PND4 offspring and dams. *Cyp2b2* expression was increased approximately 2.5- to 3-fold control in GD20 and PND22 dams. *Cyp3a1* was induced 2-fold for PND22 dams only, while *Cyp3a9* expression was induced to approximately 2-fold for GD20 dams only. These effects are consistent with our previous observations from a short-term exposure, which demonstrated that a 4-day TCS-exposure

resulted in up-regulated expression of both *Cyp2b2* and *Cyp3a1/23* (Paul et al., 2010b). Increased PROD activity in PND4 offspring and dams, and increased expression of *Cyp2b* and *Cyp3a* isoforms in dams, suggests that TCS may activate CAR and/or PXR, which also regulate the expression of key Phase II catabolic enzymes and hepatic transporters that maintain euthyroid status (Kretschmer *et al.*, 2005). Activation of these receptors could initate transcriptional up-regulation of UGTs and SULTs, thereby supporting our hypothesis that TCS-induced hypothyroxinemia *in vivo* may at least partially result from up-regulated hepatic catabolism of THs.

However, the observed effects of TCS on markers of hepatic catabolism were minor. UGT-T4 activity was only increased in PND22 dams, and the gene expression for *Ugt1a1*, *Ugt1a6, Sult1b1,* and *Sult1c3* were unchanged for all time points. UGT-T4 activity increased nearly 50% for PND22 dams only, with no effects of TCS exposure on UGT-T4 activity for PND4 offspring or GD20 dams (UGT-T4 activity was not measured in GD20 fetuses). This small increase in UGT-T4 activity was consistent with the moderate increases observed in UGT-T4 activity in rat hepatic microsomes following non-developmental 4-day (Paul et al., 2010b) and 31-day exposures (Zorrilla et al., 2009). Increased glucuronidation of T4 is thought to result in increased biliary excretion of conjugated hormone (Barter *et al.*, 1994; Liu *et al.*, 1995; Vansell *et al.*, 2002; Vansell *et al.*, 2001). PND4 neonates did demonstrate decreased serum T4 concentrations, but no detectable changes in UGT-T4 activity, but basal hepatic UGT-T4 activity may not reach adult levels until mid-lactation or PND15 (de Zwart et al., 2008). Previous work in our lab has demonstrated UGT-T4 activity was inducible with perinatal DE-71 exposure in PND4 offspring and GD20 dams (Zhou et al., 2002). Failure to observe an increase in UGT-T4 activity in PND4 offspring and GD20 dams may just be the

result of a failure to greatly perturb UGT-T4 activity, as T4 changes were moderate. Another confounding factor may be that UGT-T4 activity does not have a clear linear relationship with T4 concentrations (Craft et al., 2002; Hood *et al.*, 2000; Richardson *et al.*, 2010). To demonstrate that our UGT-T4 assay was functional, we utilized two positive control microsome pools from rats treated with TCDD/Aroclor1254, and rats treated with PB/PCN. TCDD and Aroclor1254 are aryl hydrocarbon receptor (AhR) and AhR/CAR activators, while PB can activate CAR and PXR, and PCN is an agonist for PXR. While regulation of Ugt1a1 is thought to depend on multiple receptors including CAR, PXR, and the glucocorticoid receptor (GR) (Sugatani *et al.*, 2005), Ugt1a6 is transcriptionally regulated by AhR (Auyeung *et al.*, 2003). The UGT-T4 assay is non-specific and includes Ugt1a1, 1a6, and 1a7 activities; our control data suggest that AhR activators, namely TCDD and Aroclor 1254, more successfully increase UGT-T4 activity in this assay, versus CAR/PXR activators, PB and PCN, which failed to increase UGT-T4 activity. Based on CYP induction, and a report of TCS activation of the human PXR, we hypothesize that TCS is a CAR/PXR-type activator that may not produce significant UGT-T4 activity increases in this assay. Failure of TCS to significantly increase the expression of *Ugt1a1* and *Sult1c3* is inconsistent with our previously published increases in these isoforms following non-developmental 4-day exposure to weanling rats (Paul et al., 2010b). Thus it would seem probable that TCS is a weak activator of Phase II hepatic catabolism function, and that other MOAs may contribute to the moderate hypothyroxinemia observed. Verification that the negative results for changes in gene expression for UGT and SULT isoforms were not due to inability to successfully run the low density arrays was accomplished using control mRNA from TCDD-, Aroclor1254-, PB-, and PCN-treated livers, derived from the same livers from which

microsomal pools were made, was also examined by low density qPCR array (Figure 10, B). Only PCN appeared to increase *Ug1a1* expression, while AhR activator TCDD and mixed AhR/CAR activator Aroclor 1254 induced *Ugt1a6* expression. *Sult1b1* was most highly increased by TCDD, Aroclor1254, and PCN, suggesting an AhR/PXR transcriptional regulation for this gene. *Sult1c3* expression was best increased by AhR activators TCDD and Aroclor1254. Though no dose-response information is available due to the use of a single dose for each compound, these results clearly demonstrate the inducibility of gene expression using these low density arrays.

Hepatic gene expression of markers of Phase III hepatic transport activity were also unchanged by TCS exposure for any time point, in accordance with our previous *in vivo* assessment of hepatic transporter expression following a 4-day exposure (Paul et al., 2010b). It was important to verify that that the low density arrays were executed correctly in the absence of any effect for any hepatic transporter. Prototypical nuclear receptor activators are known to increase the mRNA expression and protein content of key hepatic transporters that function in the uptake and biliary elimination of THs, including uptake transporters Oatp1a1 and Oatp1a4 and canalicular transporters Mdr1 and Mrp2 (Friesema *et al.*, 1999). The well-known CAR activator, PB, has been shown to increase the expression and protein content of canalicular transporters Mdr1 and Mrp2 in rats and human hepatocytes (Jigorel *et al.*, 2006; Johnson *et al.*, 2002). Prototypical PXR agonist PCN has been shown to increase Oatp1a4 mRNA in mouse liver (Cheng *et al.*, 2005) and Mrp2 protein expression in rat liver (Johnson et al., 2002), and another PXR agonist, dexamethasone, is also known to increase hepatic transporter expression and function of Oatp1a4 and Mrp2 (Turncliff *et al.*, 2004). To demonstrate that increases in hepatic transporter mRNA expression were possible with

prototypical inducers, mRNA from TCDD-, Aroclor1254-, PB-, and PCN-treated livers, derived from the same livers from which microsomal pools were made, was also examined by low density qPCR array. Though no dose-response relationships can be established since single treatment groups by chemical were employed, PCN, the PXR agonist, appeared to increase the expression Mrp2 by the largest margin, with moderate increases observed with all of the inducers. PCN was the only control compound to induce Oatp1a4 expression. These results with positive control compounds suggest that activation of AhR, CAR, and in particular PXR, can up-regulated expression of these targets.

Hepatic gene expression of markers of liver-specific TH-responsive genes demonstrated no effects of TCS treatment, suggesting that TCS did not change the T3 concentrations at the target tissue. Genes defined as TH-responsive for this work are deiodinase I (*DioI*), malic enzyme I (*MeI*), and Spot14 (*Thrsp*). Hepatic expression of *MeI* has been shown to increase with TH-disrupting chemicals, including perfluorooctanesulfonate (PFOS) (Chang *et al.*, 2008) and polychlorinated biphenyls (PCBs) (Gauger *et al.*, 2007) with concomitant serum T4 decreases. Spot 14/Thrsp protein is thought to be regulated by CAR (Breuker et al 2010). Again the lack of effects with TCS exposure required verification that these assays were successful. The expression of *DioI* and *Thrsp* seemed unaffected by any of the controls employed, but *MeI* expression was induced greatly by AhR activators TCDD and Aroclor 1254, with good demonstrated increases with PB and PCN of a slightly decreased magnitude.

Together these data indicate that TCS is capable of mildly decreasing serum T4 concentrations in dams, fetuses, and young neonates, and that transplacental exposure provides more TCS to the progeny than lactation exposure. Further, minor increases in markers of Phase I and Phase II catabolism suggest that TCS may activate hepatic nuclear

receptors, e.g. CAR and/or PXR, to transcriptionally up-regulate metabolism of THs to result in hypothyroxinemia.

6. Conclusion

This study confirmed previous observations of moderate hypothyroxinemia in dams and young neonates induced by TCS exposure. This study provided the first report demonstrating the importance of transplacental exposure and effects on GD20 T4 in dams and offspring, and established that exposure conveyed from dams to offspring diminishes over the lactation period, such that PND14 and PND21 neonatal T4 concentrations return to control levels, likely due to a lack of TCS exposure. Further, this study provides some evidence that TCS up-regulated hepatic catabolism in GD20 and PND22 dams and PND4 offspring, though the effects were minor. The minor effects on CYP and UGT activity and expression are consistent with previous findings in a short-term exposure model that suggest that TCS activates CAR and/or PXR resulting in downstream up-regulation of hepatic catabolism of thyroid hormones. The relatively minor effects on Phase I and Phase II metabolism are in keeping with the mild hypothyroxinemia induced as a result of treatment. Further research should characterize the human relevance of this work; in particular, the proposed MOA should be evaluated in a human model because maternal hypothyroxinemia, however minor, indicates a potential hazard for human neurodevelopment. Specifically, the putative initiating key event, interaction with CAR and/or PXR, is a highly species-dependent event and should be evaluated with human receptors.

7. Acknowledgements

The authors would like to thank Willard Anderson and Kateland Grant for assistance during animal sacrifice; Angela Buckalew and Ashley Murr for assistance with the TSH

assay; and Elizabeth Boykin and Dr. Geremy Knapp for helpful qPCR training. Triclosan was a generous gift from Drs. James Plautz and Lisa Navarro of Ciba Specialty Chemicals. Raw data files can be obtained by contacting the corresponding author.

8. Funding

K.B. Paul was funded by a PhRMA Foundation Predoctoral Pharmacology/Toxicology Fellowship, the EPA/UNC Toxicology Research Program Training Agreement (CR833237), and the National Institute of Environmental Health Science Training Grant (T32-ES07126) during this work. This work was partially supported by a cooperative research and development agreement (CRADA) between the U.S. EPA and BASF Corporation (CRADA No. 546-09).

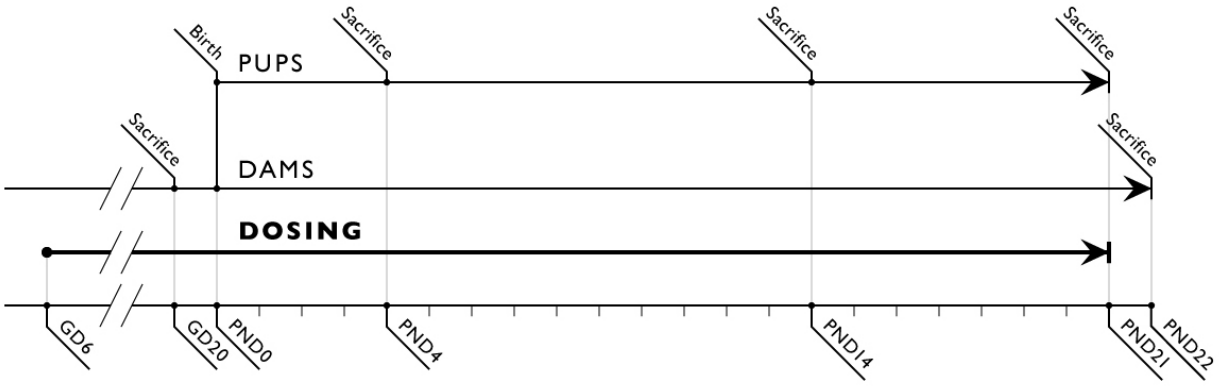


Figure 4.1. Dosing and tissue collection schedule, from GD6 through PND22. Dams received TCS by oral gavage daily; no TCS was administered directly to offspring. Serum and liver were collected prenatally on GD20 from dams and fetuses and postnatally on PND4, PND14, and PND21 from pups and on PND22 from dams.

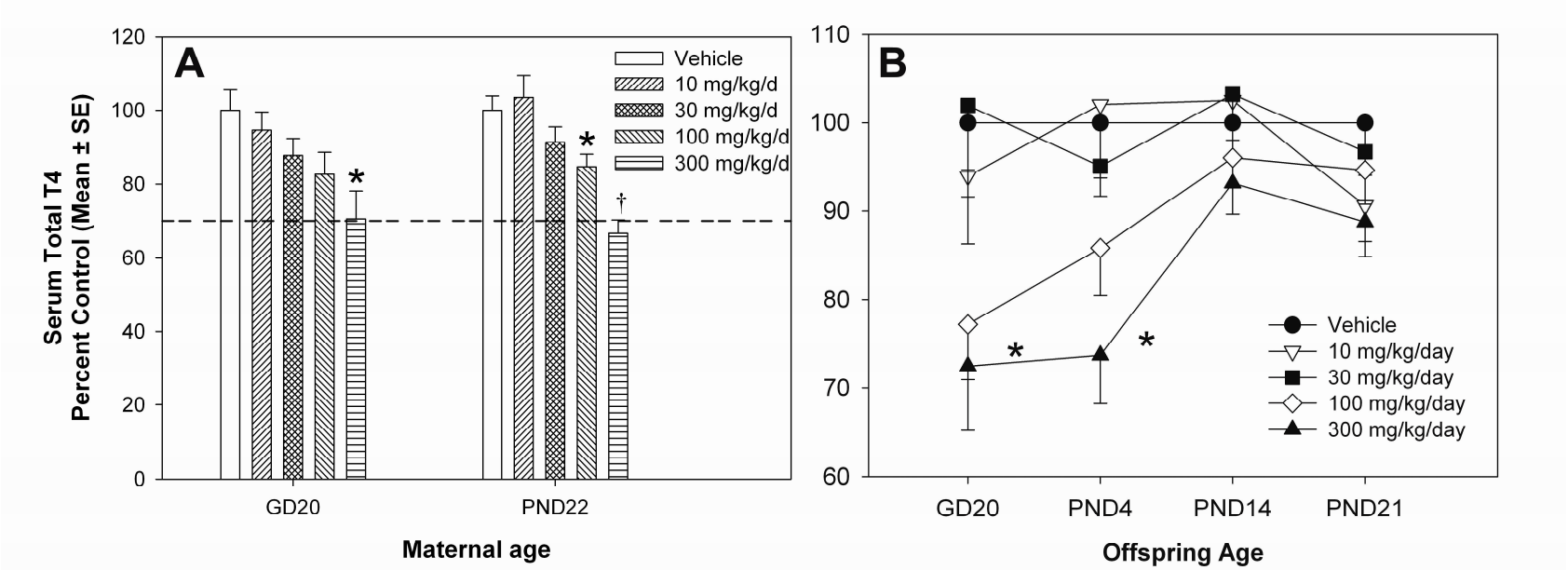


Figure 4.2. Percent of control T4. A) GD20 and PND22 Dams, with vehicle control = 23.1 ± 1.3 and 51.8 ± 2.1, respectively. B) GD20 Fetuses and Pups aged PND4, PND14, and PND21, with vehicle control = 4.15 ± 0.22, 9.70 ± 0.43, 42.8 ± 1.9, 38.2 ± 1.3 ng/mL, respectively. V=vehicle control; † = significantly different from control and all other treatment groups; * = significantly different from vehicle control.

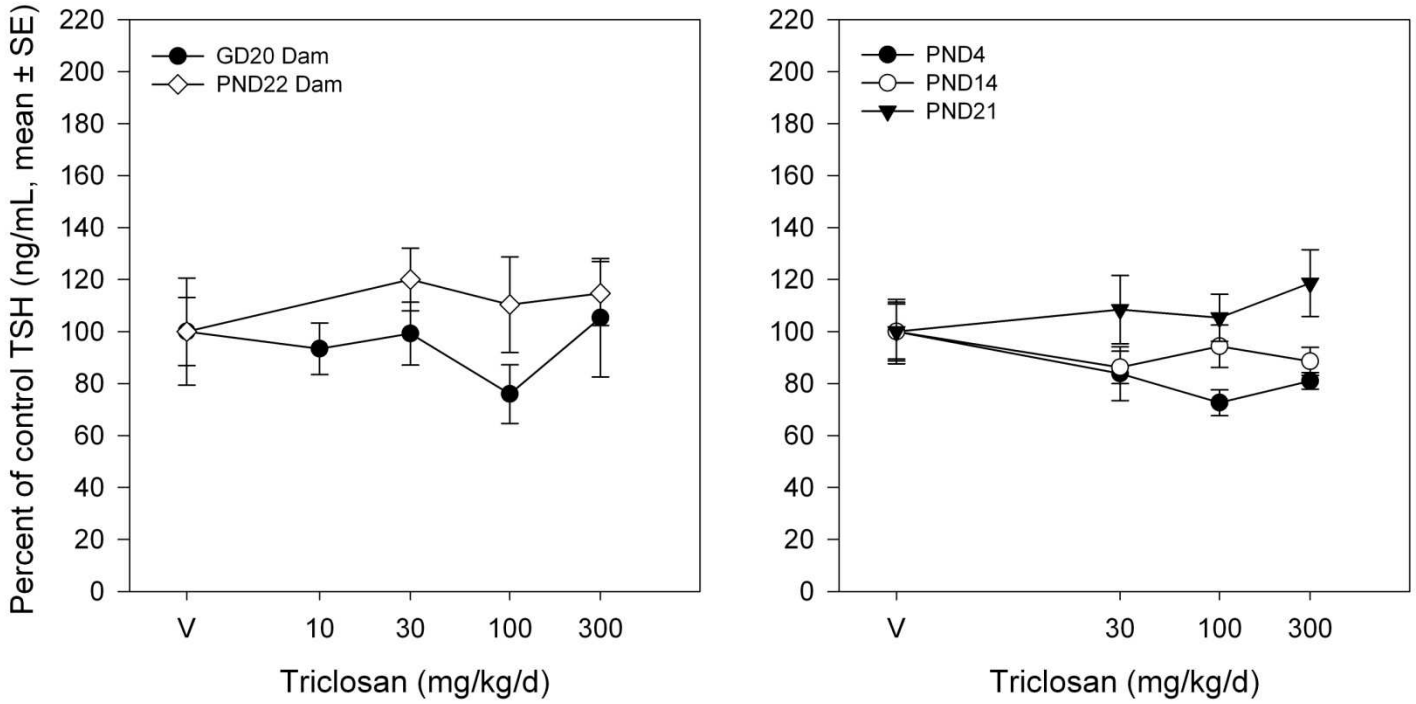


Figure 4.3. Percent of control serum TSH for dams and offspring. A) GD20 and PND22 Dams, with vehicle control = 3.51 ± 0.46 and 3.28 ± 0.68 ng/mL, respectively. B) Pups aged PND4, PND14, and PND21, with vehicle control = 2.10 ± 0.22, 2.19 ± 0.27, 1.73 ± 0.20 ng/mL, respectively. V=vehicle control.

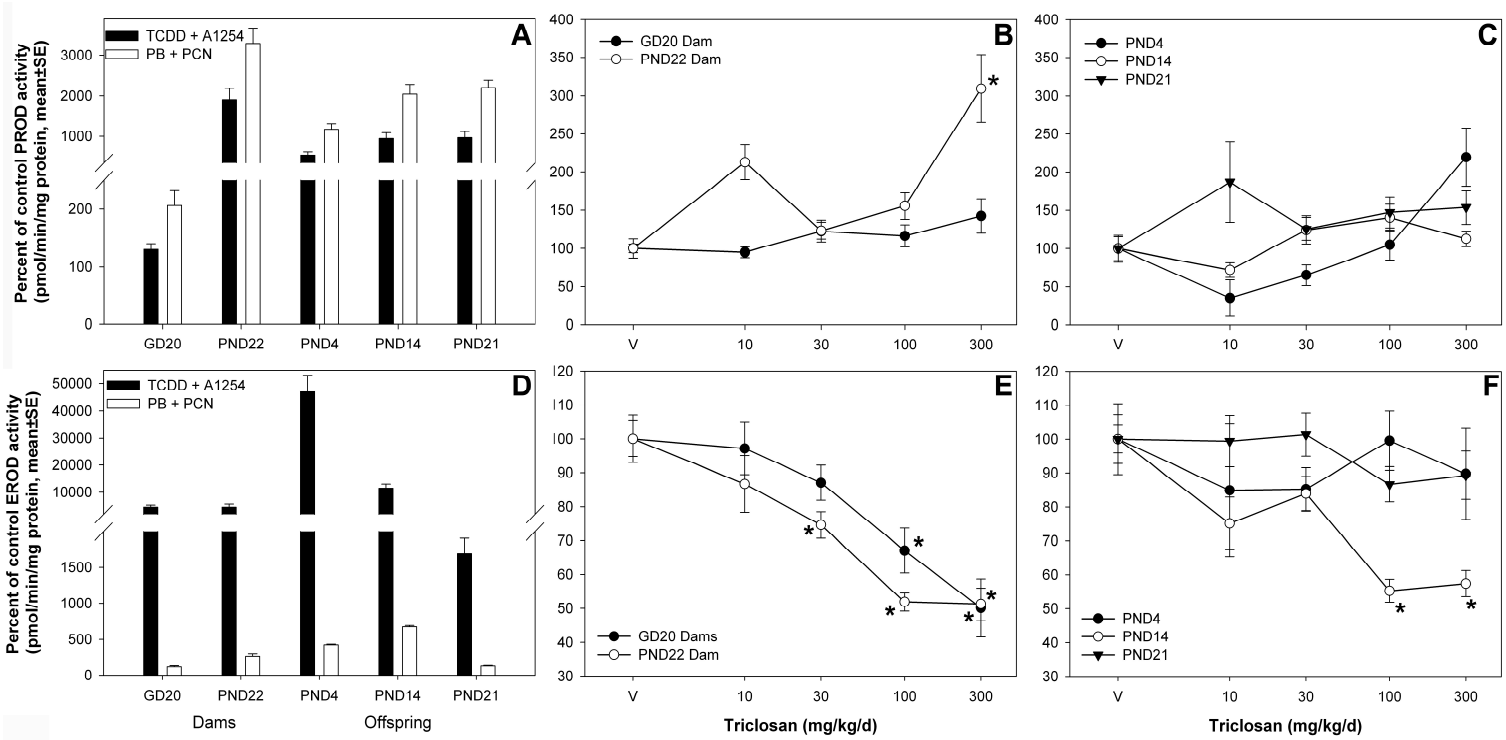


Figure 4.4. Percent of control EROD and PROD activity for dams and offspring compared to controls. A) PROD activity for control microsome pools. The controls are presented as the percent of vehicle control for each age tested. TCDD+ A1254 is a microsome pool from rats exposed to a single gavage dose of TCDD (10 µg/kg) or Aroclor 1254 (300 mg/kg); PB+PCN is a microsome pool from rats that received a 4-day intraperitoneal exposure to phenobarbital (81 mg/kg/d) or PCN (50 mg/kg). B) PROD activity for dams at GD20 and PND22. C) PROD activity for pups aged PND4, PND14, and PND21. D) EROD activity for controls. E) EROD activity for dams at GD20 and PND22. F) EROD activity for pups aged PND4, PND14, and PND21.V=vehicle control; * = significantly different from vehicle control.

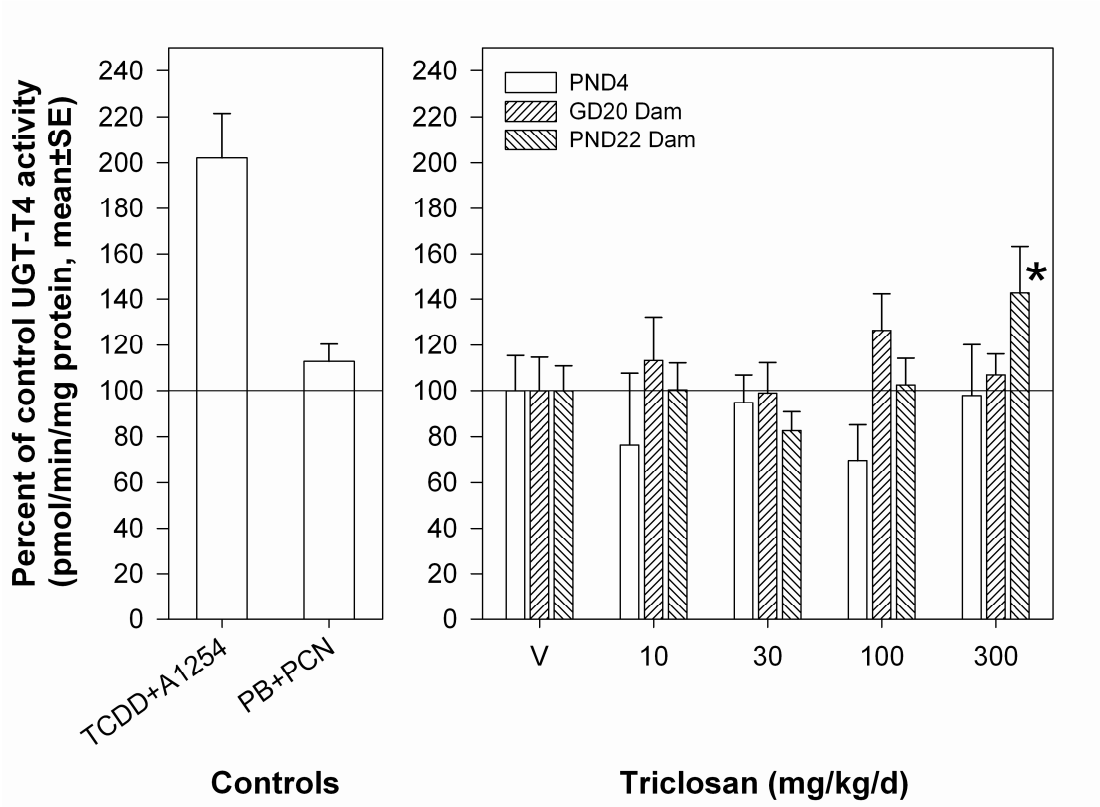


Figure 4.5. Hepatic microsomal UGT-T4 activity. A) Positive controls. TCDD+ A1254 is a microsome pool from rats exposed to a single gavage dose of TCDD (10 µg/kg) or Aroclor 1254 (300 mg/kg); PB+PCN is a microsome pool from rats that received a 4-day intraperitoneal exposure to phenobarbital (81 mg/kg/d) or PCN (50 mg/kg). B) PND4 neonates, GD20 dams, and PND22 dams. V=vehicle control; * = significantly different from vehicle control.

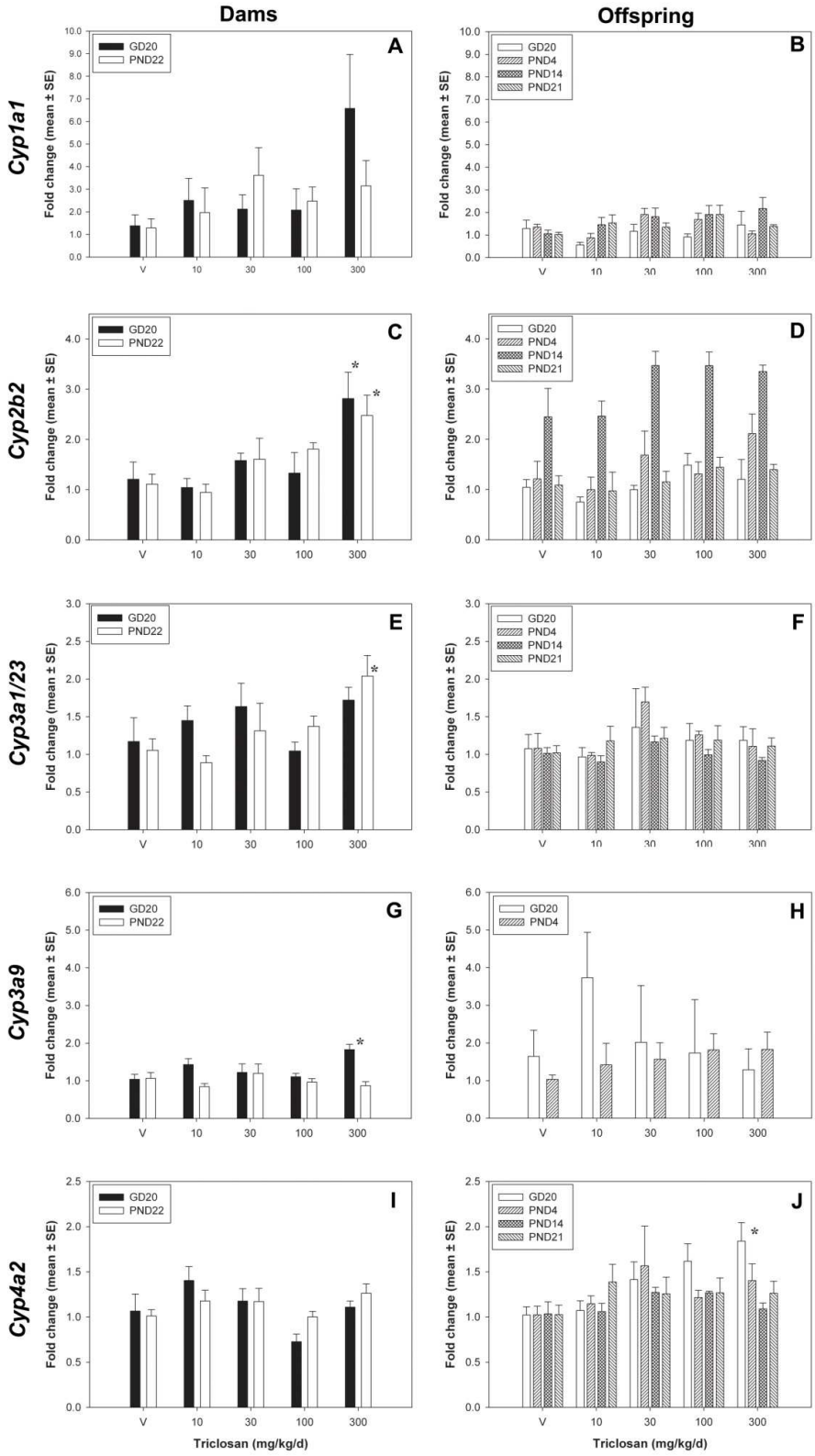

Dams
Offspring
Cyp1a1
Cyp2b2
Cyp3a1/23
Cyp3a9
Cyp4a2
A
B
C
D
E
F
G
H
I
J
GD20
PND22
PND4
PND14
PND21
Fold change (mean ± SE)
V
10
30
100
300
Triclosan (mg/kg/d)

Figure 4.6. Hepatic CYP gene expression changes. A,C,E,and G) Dams: GD20 and PND22 dams for Cyp1a1, Cyp2b2, Cyp3a1/23, Cyp3a2, Cyp3a9, and Cyp4a2. B, D, F, and I) Offspring: GD20 fetuses, PND4 , PND14, and PND21 pups for Cyp1a1, Cyp2b2, Cyp3a1/23, Cyp3a2, Cyp3a9, and Cyp4a2. V=vehicle control.* = significantly different from vehicle control.

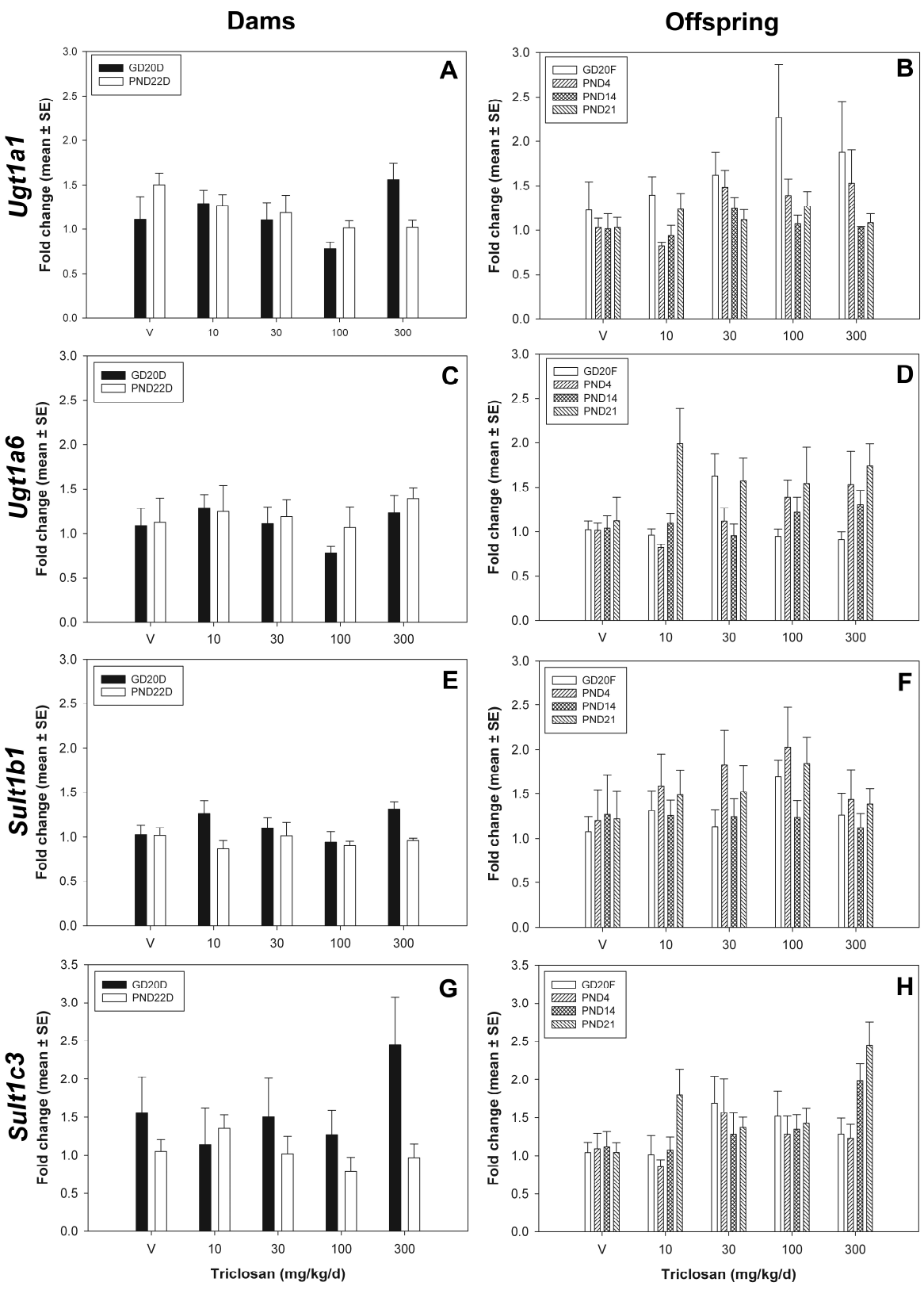

Dams
Offspring
Ugt1a1
Ugt1a6
Sult1b1
Sult1c3
Fold change (mean ± SE)
GD20D
PND22D
GD20F
PND4
PND14
PND21
A
B
C
D
E
F
G
H
V
10
30
100
300
Triclosan (mg/kg/d)

Figure 4.7. Hepatic Phase II gene expression. A,C,E,and G) Dams: GD20 and PND22 dams for Ugt1a1, Ugt1a6, Sult1b1, Sult1c3. B, D, F, and I) Offspring: GD20 fetuses, PND4 , PND14, and PND21 pups for Ugt1a1, Ugt1a6, Sult1b1, Sult1c3. V=vehicle control.

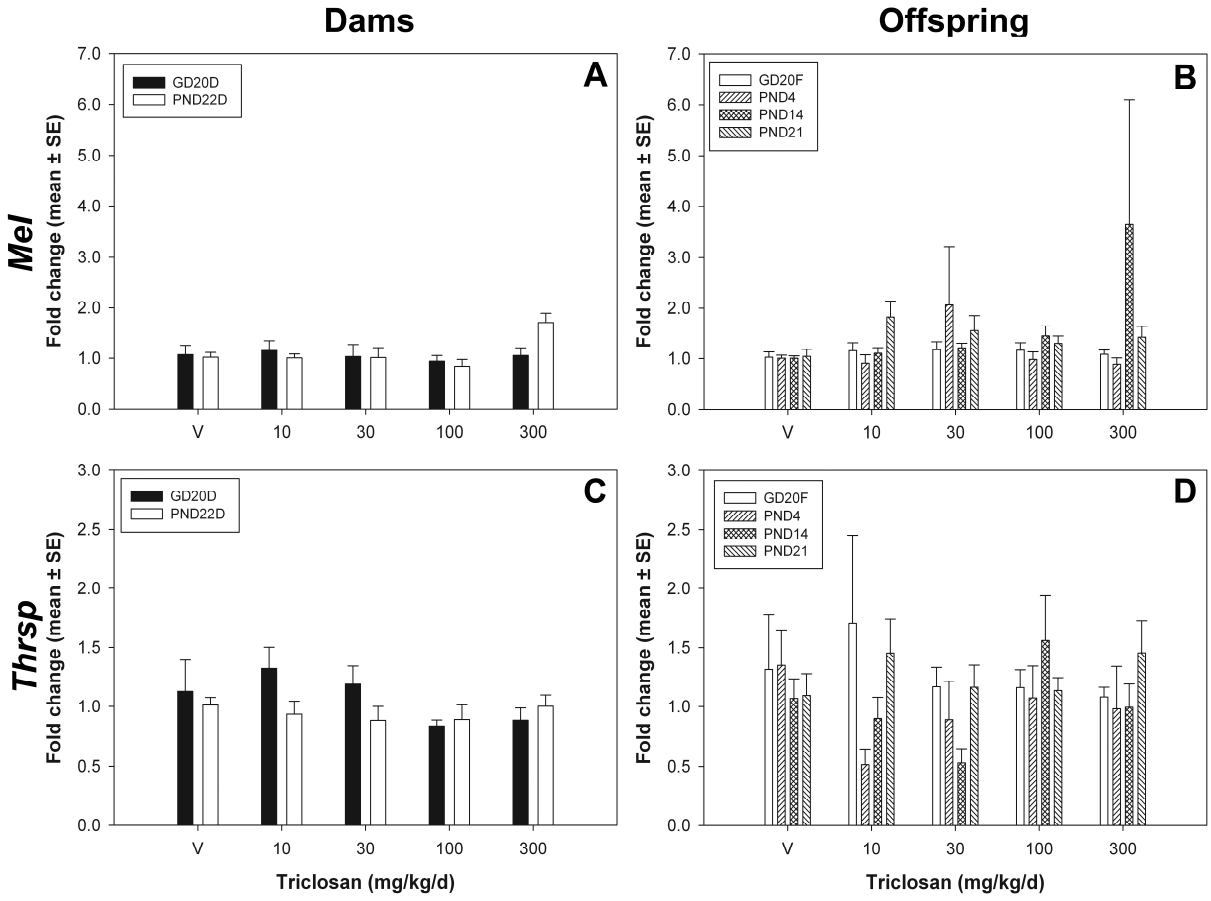


Figure 4.8. Hepatic TH-responsive gene expression. A and C) Dams: GD20 and PND22 dams for MeI and Thrsp. B and D) Offspring: GD20 fetuses, PND4 , PND14, and PND21 pups for MeI and Thrsp. V=vehicle control.

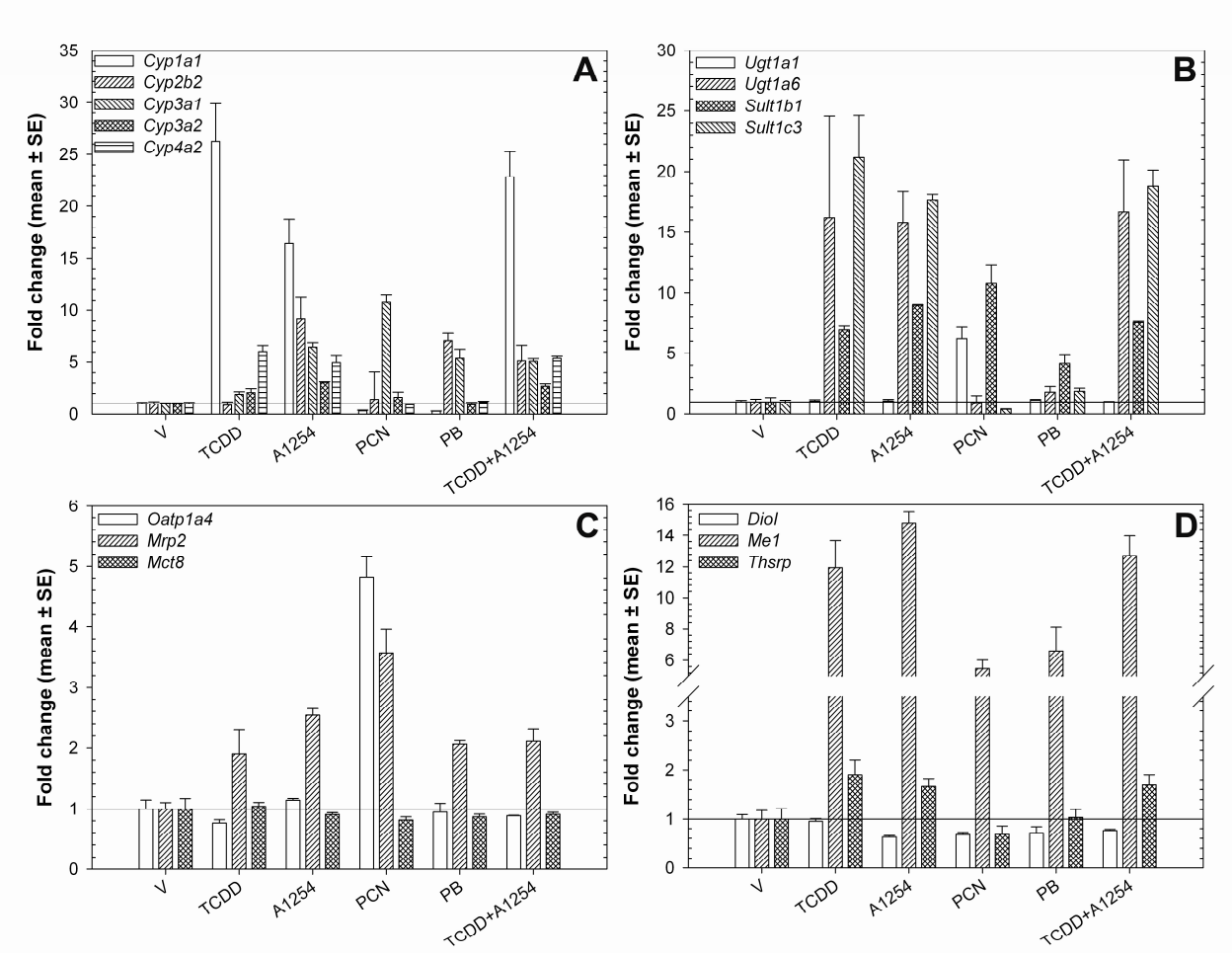


Figure 4.9. Positive controls (Phase I, Phase II, Phase III, and TH-sensitive). A) Phase I metabolic genes: Cyp1a1, Cyp2b2, Cyp3a1/23, Cyp3a2, Cyp3a9, and Cyp4a2. B) Phase II metabolic genes; Ugt1a1, Ugt1a6, Sult1b1, and Sult1c3. C) Hepatic transport genes: Oatp1a4, Mrp2, Mct8. D) TH-responsive genes: DioI, MeI, and Thrsp. V = vehicle control; TCDD = hepatic mRNA from rats exposed to a single gavage dose of TCDD (10 µg/kg); A1254 = hepatic mRNA from rats exposed to single 300 mg/kg dose of Aroclor 1254; PB = hepatic mRNA from rats that received a 4-day intraperitoneal exposure to PB (81 mg/kg/d); PCN = hepatic mRNA from rats that received a 4-day intraperitoneal exposure to PCN (50 mg/kg).

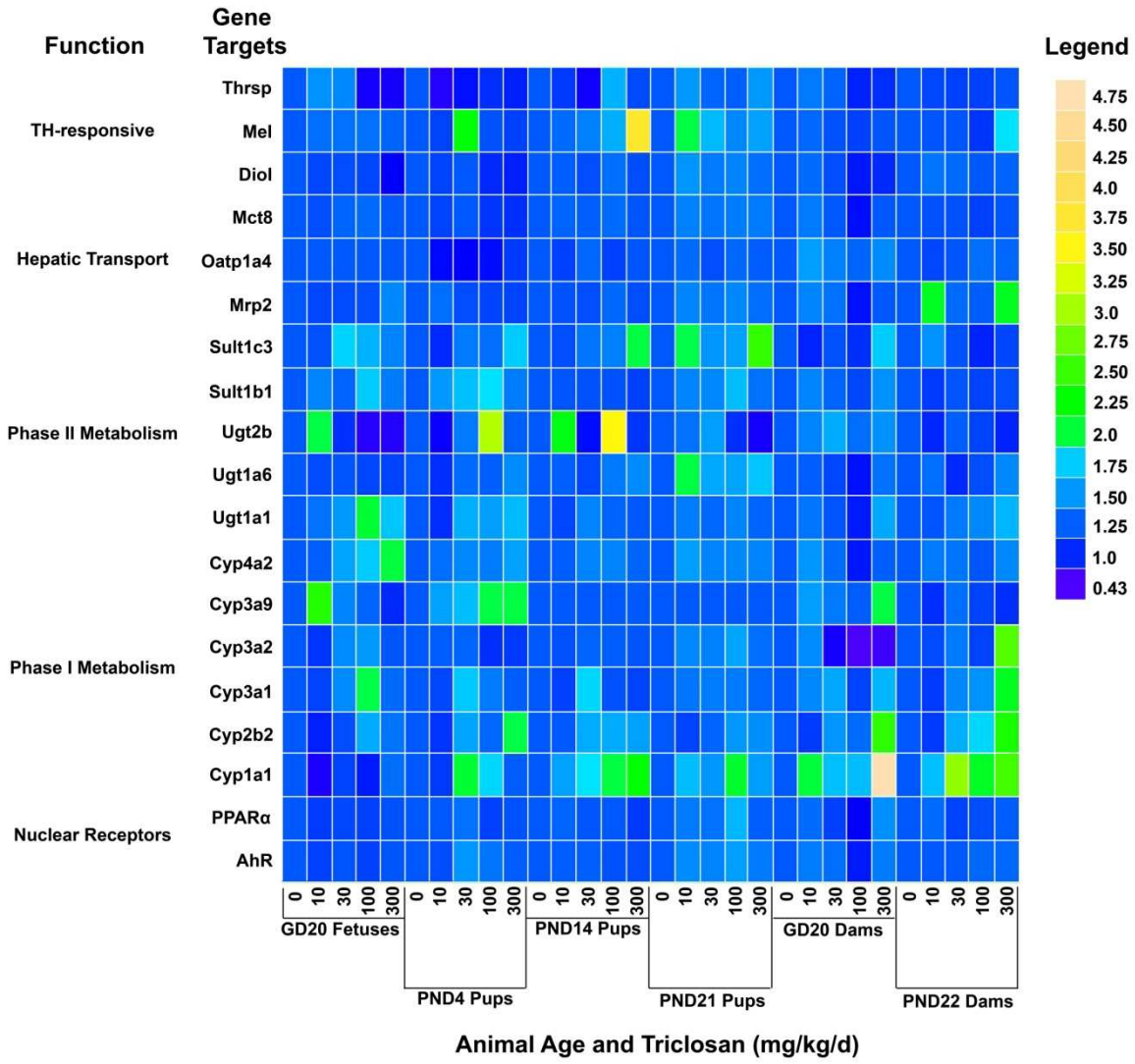
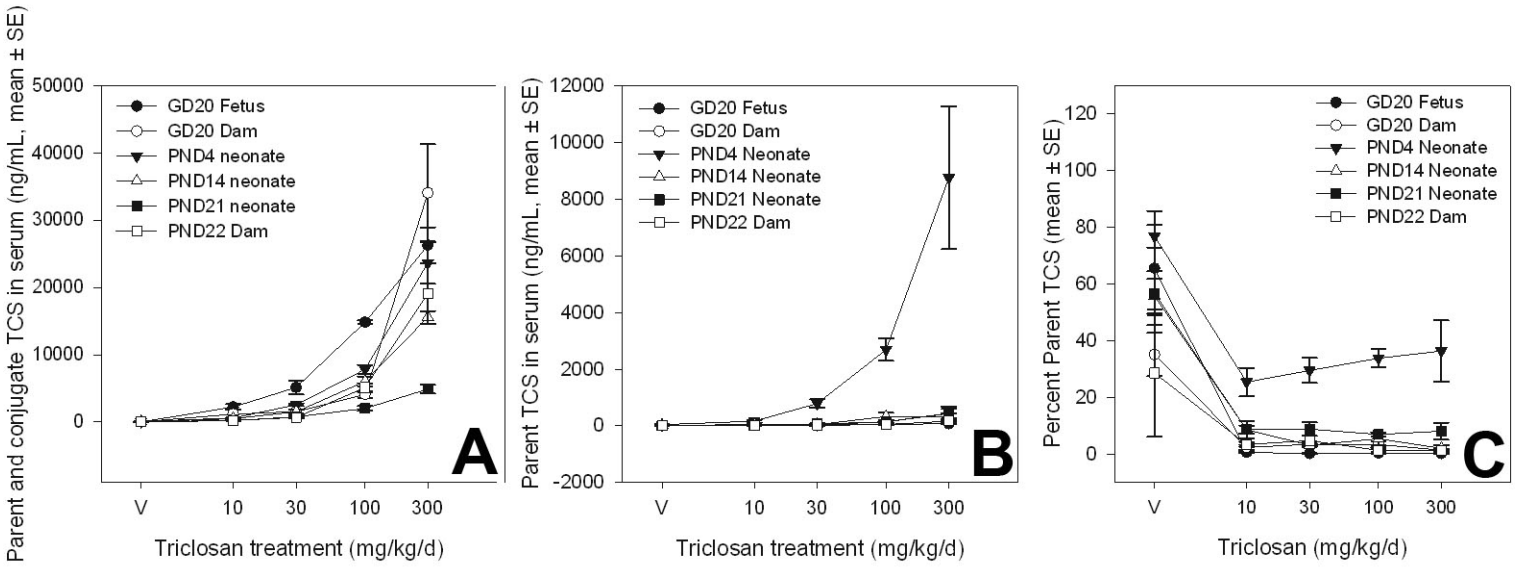


Figure 4.10. Heatmap of hepatic gene expression by time point and TCS exposure group (mg/kg/day). For all time points, the vehicle control value was standardized as 1, and all of the other exposure groups are expressed as a ratio of the vehicle control. Heatmap was generated without scaling by column or row, as data were already standardized to the vehicle control (generated with R 2.12.0).


Parent and conjugate TCS in serum (ng/mL, mean ± SE)
50000
40000
30000
20000
10000
0
GD20 Fetus
GD20 Dam
PND4 neonate
PND14 neonate
PND21 neonate
PND22 Dam
A
V
10
30
100
300
Triclosan treatment (mg/kg/d)
Parent TCS in serum (ng/mL, mean ± SE)
12000
10000
8000
6000
4000
2000
0
-2000
GD20 Fetus
GD20 Dam
PND4 Neonate
PND14 Neonate
PND21 Neonate
PND22 Dam
B
V
10
30
100
300
Triclosan treatment (mg/kg/d)
Percent Parent TCS (mean ± SE)
120
100
80
60
40
20
0
GD20 Fetus
GD20 Dam
PND4 Neonate
PND14 Neonate
PND21 Neonate
PND22 Dam
C
V
10
30
100
300
Triclosan (mg/kg/d)

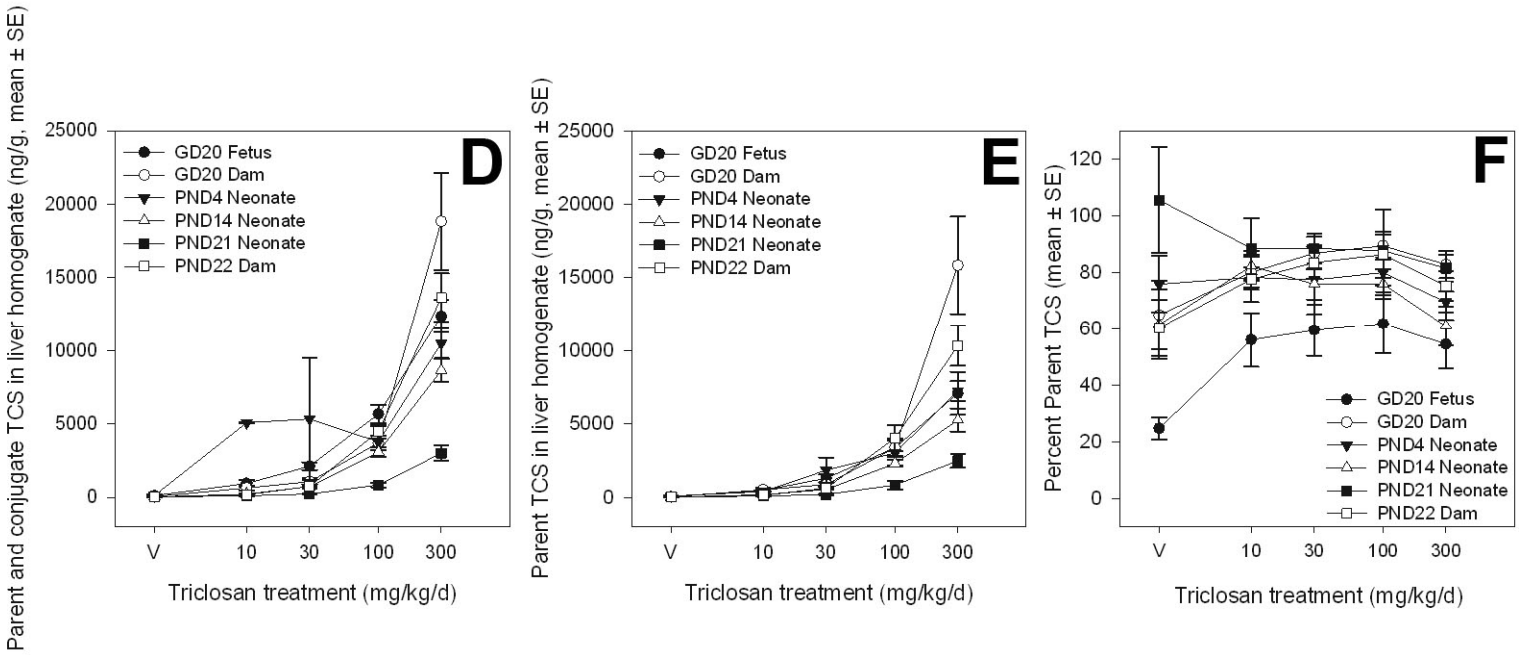


Figure 4.11. Mean total, parent, and percent parent TCS content in sera and liver (ng/mL, ± SE). A) Total TCS in sera, comprised of parent and glucuronide and sulfate conjugates (ng/mL); B) Parent TCS only in sera (ng/mL); C) Percent parent out of the total TCS in sera; D) Total TCS in liver, comprised of parent and glucuronide and sulfate conjugates (ng/g); E) Parent TCS only in liver (ng/g); F) Percent parent out of the total TCS in liver.

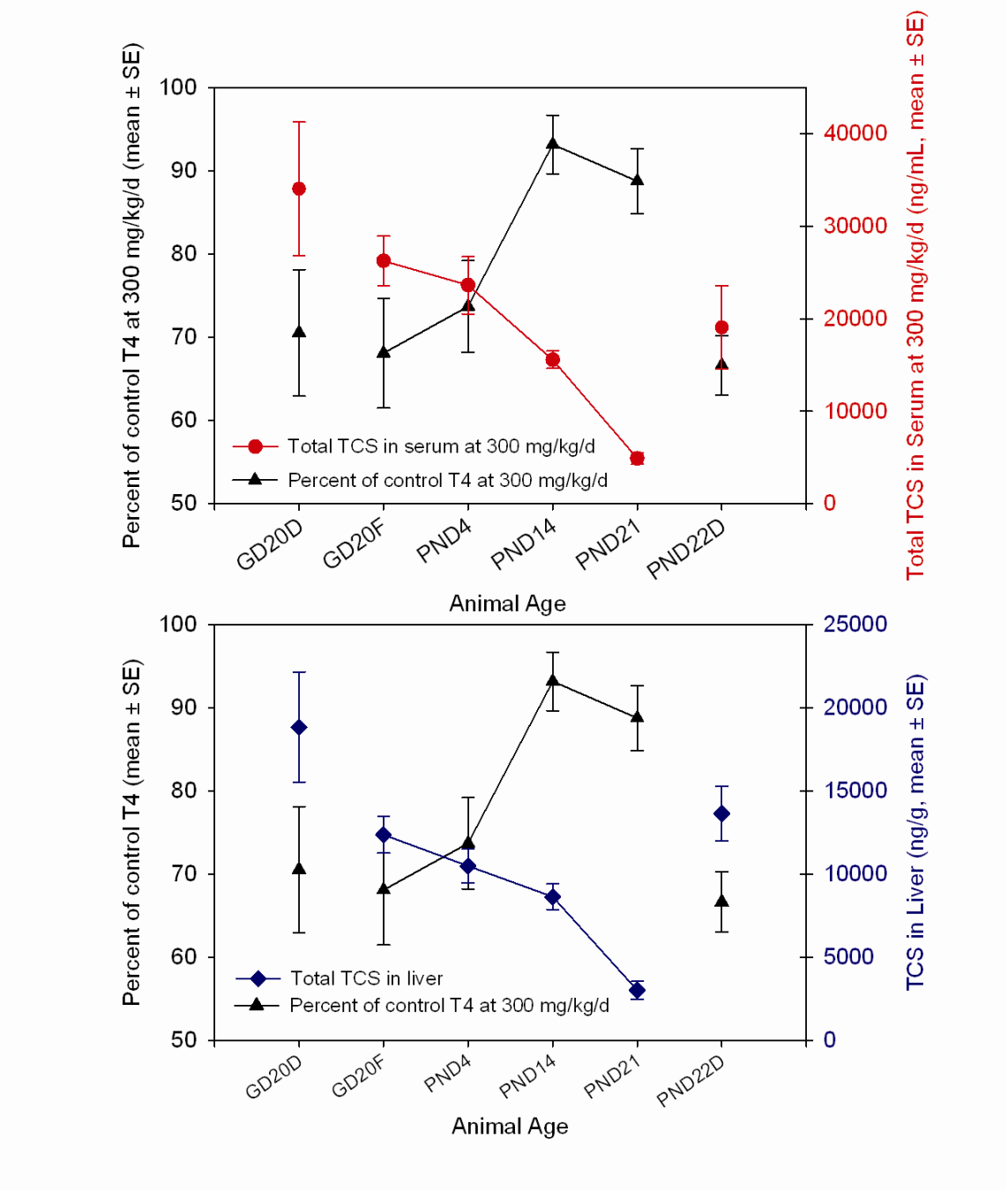


Figure 4.12. TCS content in sera and liver versus serum T4 concentrations at 300 mg/kg/day. A) Total TCS in sera, comprised of parent and glucuronide and sulfate conjugates (ng/mL); B) Parent TCS only in sera (ng/mL); C) Percent parent out of the total TCS in sera; D) Total TCS in liver, comprised of parent and glucuronide and sulfate conjugates (ng/g); E) Parent TCS only in liver (ng/g); F) Percent parent out of the total TCS in liver.

Experimental Block	Time Points	T4	T3	TSH	EROD/PROD UGT-T4	qPCR	Analytical measurement
1	Offspring: PND4, 14, 21; Dams: PND22	n = 21, 12, 22, 22, 18 for 0, 10, 30, 100, 300 mg/kg/d	Not measured	n = 10, 10, 10, 8 for 0, 30, 100, 300 mg/kg/d	n = 21, 12, 22, 22, 18 for 0, 10, 30, 100, 300 mg/kg/d	Not measured	Not measured
2			n = 11, 12, 12, 12, 10 for 0, 10, 30, 100, 300 mg/kg/d	Not measured		n = 6/ treatment group	n = 6/ treatment group
3	Fetuses and Dams: GD20	n = 11, 11, 11, 11, 10 for 0, 10, 30, 100, 300 mg/kg/d				n = 6/ treatment group	n = 6/ treatment group

Table 4.1. Experimental blocks and n for each parameter measured. Experimental Blocks 1 and 2 included postnatal time points and experimental Block 3 included a single prenatal time point.

Table 4.2. Taqman Gene Expression Assays included in the Taqman Custom Low Density Array. Rps18 was used as the endogenous control to standardize the gene expression assay results for GD20 and PND22 dams, GD20 fetuses, and PND21 offspring. PND4 and PND14 offspring were standardized to Actb.

Summary of Taqman Gene Expression Assays included in the Taqman Custom Low Density Array			
Function category	Gene	Gene abbreviation	Taqman Gene Expression Assay ID
Nuclear receptors	aryl-hydrocarbon receptor	AhR	Rn00565750_m1
	peroxisome proliferator activated receptor alpha	Pparα	Rn00566193_m1
Phase I metabolism	cytochrome P450, family 1, subfamily a, polypeptide 1	Cyp1a1	Rn00487218_m1
	cytochrome P450, family 2, subfamily b, polypeptide 2	Cyp2b2	Rn02786833_m1
	cytochrome P450, family 3, subfamily a, polypeptide 23/polypeptide 1	Cyp3a1/3a23	Rn01640761_gH
	cytochrome P450, family 3, subfamily a, polypeptide 2	Cyp3a2	Rn01412889_mH
	cytochrome P450, family 3, subfamily a, polypeptide 9	Cyp3a9	Rn00595977_m1
	cytochrome P450, family 4, subfamily a, polypeptide 2	Cyp4a2	Rn01417066_m1
Phase II metabolism	UDP glucuronosyltransferase 1 family, polypeptide A1	Ugt1a1	Rn00754947_m1
	UDP glucuronosyltransferase 1 family, polypeptide A6	Ugt1a6	Rn00756113_mH
	UDP glycosyltransferase 2 family, polypeptide B	Ugt2b	Rn02349652_m1
	sulfotransferase family, cytosolic, 1B, member 1	Sult1b1	Rn00673872_m1
	sulfotransferase family, cytosolic, 1C, member 3	Sult1c3	Rn00581955_m1
Hepatic transport	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Abcc2/Mrp2	Rn00563231_m1
	solute carrier organic anion transporter family, member 1a4	Slco1a4/Oatp1a4	Rn00756233_m1
	solute carrier family 16 (monocarboxylic acid transporters), member 2	Slc16a2/Mct8	Mm00486202_m1
Thyroid hormone-responsive	deiodinase, iodothyronine, type I	Dio1	Rn00572183_m1
	malic enzyme 1 NADP(+)-dependent, cytosolic	Me1	Rn00561502_m1
	thyroid hormone responsive	Thrsp	Rn01511034_m1
Candidate endogenous controls	ribosomal protein L13A	Rpl13a	Rn00821946_g1
	ribosomal protein S18	Rps18	Rn01428915_g1
	glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Rn99999916_s1
	actin, beta	Actb	Rn00667869_m1

No-observed-effect level and benchmark dose levels for T4 decreases by age			
Age	NOEL	BMD	BMDL
GD20 Dams	100	124	25.2
PND22 Dams	30	115	62.1
GD20 Fetuses	100	95.4	33.0
PND4 Pups	100	150	61.8

Table 4.3. No-observed-effect level (NOEL) and benchmark dose levels for T4 decreases. NOELs are reported as the mg/kg/day exposure group. BMD = benchmark dose US EPA Benchmark Dose Software (BMDS Version 2.0Beta) was used to determine the BMD and BMDL, with a 95% confidence limit, for the selected BMR = 20% reduction in serum T4.

References

Adolfsson-Erici, M., M. Pettersson, J. Parkkonen and J. Sturve (2002). Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**, 1485-1489.

Allmyr, M., M. Adolfsson-Erici, M. S. McLachlan and G. Sandborgh-Englund (2006a). Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *The Science of the total environment* **372**, 87-93.

Allmyr, M., M. S. McLachlan, G. Sandborgh-Englund and M. Adolfsson-Erici (2006b). Determination of triclosan as its pentafluorobenzoyl ester in human plasma and milk using electron capture negative ionization mass spectrometry. *Analytical chemistry* **78**, 6542-6546.

Allmyr, M., F. Harden, L. M. Toms, J. F. Mueller, M. S. McLachlan, M. Adolfsson-Erici and G. Sandborgh-Englund (2008). The influence of age and gender on triclosan concentrations in Australian human blood serum. *The Science of the total environment* **393**, 162-167.

Allmyr, M., G. Panagiotidis, E. Sparve, U. Diczfalusy and G. Sandborgh-Englund (2009). Human exposure to triclosan via toothpaste does not change CYP3A4 activity or plasma concentrations of thyroid hormones. *Basic Clin Pharmacol Toxicol* **105**, 339-344.

Auyeung, D. J., F. K. Kessler and J. K. Ritter (2003). Mechanism of rat UDP-glucuronosyltransferase 1A6 induction by oltipraz: evidence for a contribution of the Aryl hydrocarbon receptor pathway. *Molecular pharmacology* **63**, 119-127.

Bansal, R., S. H. You, C. T. Herzig and R. T. Zoeller (2005). Maternal thyroid hormone increases HES expression in the fetal rat brain: an effect mimicked by exposure to a mixture of polychlorinated biphenyls (PCBs). *Brain Res Dev Brain Res* **156**, 13-22.

Barter, R. A. and C. D. Klaassen (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. *Toxicology and applied pharmacology* **128**, 9-17.

Beetstra, J. B., J. G. van Engelen, P. Karels, H. J. van der Hoek, M. de Jong, R. Docter, E. P. Krenning, G. Hennemann, A. Brouwer and T. J. Visser (1991). Thyroxine and 3,3',5-triiodothyronine are glucuronidated in rat liver by different uridine diphosphate-glucuronyltransferases. *Endocrinology* **128**, 741-746.

Berbel, P., J. L. Mestre, A. Santamaria, I. Palazon, A. Franco, M. Graells, A. Gonzalez-Torga and G. M. de Escobar (2009). Delayed neurobehavioral development in children born to pregnant women with mild hypothyroxinemia during the first month of gestation: the importance of early iodine supplementation. *Thyroid* **19**, 511-519.

Berbel, P., D. Navarro, E. Auso, E. Varea, A. E. Rodriguez, J. J. Ballesta, M. Salinas, E. Flores, C. C. Faura and G. M. de Escobar (2010). Role of late maternal thyroid hormones in cerebral cortex development: an experimental model for human prematurity. *Cereb Cortex* **20**, 1462-1475.

Bernal, J. (2007). Thyroid hormone receptors in brain development and function. *Nat Clin Pract Endocrinol Metab* **3**, 249-259.

Borlakoglu, J. T., A. Scott, C. J. Henderson and C. R. Wolf (1993). Expression of P450 isoenzymes during rat liver organogenesis. *Int J Biochem* **25**, 1659-1668.

Butte, N. F., C. Garza, E. O. Smith and B. L. Nichols (1984). Human milk intake and growth in exclusively breast-fed infants. *J Pediatr* **104**, 187-195.

Butte, N. F., M. G. Lopez-Alarcon and C. Garza (2002). Nutrient adequacy of exclusive breast feeding for the term infant during the first 6 months of life. *WHO* **Geneva**, 1-49.

Calafat, A. M., X. Ye, L. Y. Wong, J. A. Reidy and L. L. Needham (2008). Urinary concentrations of triclosan in the U.S. population: 2003-2004. *Environmental health perspectives* **116**, 303-307.

Chang, S. C., J. R. Thibodeaux, M. L. Eastvold, D. J. Ehresman, J. A. Bjork, J. W. Froehlich, C. Lau, R. J. Singh, K. B. Wallace and J. L. Butenhoff (2008). Thyroid hormone status and pituitary function in adult rats given oral doses of perfluorooctanesulfonate (PFOS). *Toxicology* **243**, 330-339.

Cheng, X., J. Maher, M. Z. Dieter and C. D. Klaassen (2005). Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1276-1282.

Craft, E. S., M. J. DeVito and K. M. Crofton (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**, 372-380.

Crofton, K. M., P. R. Kodavanti, E. C. Derr-Yellin, A. C. Casey and L. S. Kehn (2000). PCBs, thyroid hormones, and ototoxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. *Toxicol Sci* **57**, 131-140.

Crofton, K. M., K. B. Paul, J. M. Hedge and M. J. DeVito (2007). Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine. *Environ Toxicol Pharmacol* **24**, 194-197.

Dayan, A. D. (2007). Risk assessment of triclosan [Irgasan((R))] in human breast milk. *Food Chem Toxicol* **45**, 125-129.

de Zwart, L., M. Scholten, J. G. Monbaliu, P. P. Annaert, J. M. Van Houdt, I. Van den Wyngaert, L. M. De Schaepdrijver, G. P. Bailey, T. P. Coogan, W. C. Coussement and G. S. Mannens (2008). The ontogeny of drug metabolizing enzymes and transporters in the rat. *Reprod Toxicol* **26**, 220-230.

DeVito, M. J., W. E. Maier, J. J. Diliberto and L. S. Birnbaum (1993). Comparative Ability of Various PCBs , PCDFs, and TCDD to INduce Cytochrome P450 1A1 and 1a2 Activity Following 4 Weeks of Treatment. *Fundamental and Applied Toxicology* **20**, 125-130.

Dohler, K. D., C. C. Wong and A. von zur Muhlen (1979). The rat as model for the study of drug effects on thyroid function: consideration of methodological problems. *Pharmacol Ther B* **5**, 305-318.

Dunn, R. T., 2nd and C. D. Klaassen (1998). Tissue-specific expression of rat sulfotransferase messenger RNAs. *Drug metabolism and disposition: the biological fate of chemicals* **26**, 598-604.

Ejiri, N., K. Katayama, N. Kiyosawa, Y. Baba and K. Doi (2005a). Microarray analysis on CYPs expression in pregnant rats after treatment with pregnenolone-16alpha-carbonitrile and phenobarbital. *Exp Mol Pathol* **78**, 71-77.

Ejiri, N., K. Katayama, N. Kiyosawa, Y. Baba and K. Doi (2005b). Microarray analysis on Phase II drug metabolizing enzymes expression in pregnant rats after treatment with pregnenolone-16alpha-carbonitrile or phenobarbital. *Exp Mol Pathol* **79**, 272-277.

EPA, U. S. (1997). Exposure Factors Handbook Vol II Food Ingestion Factors. *US Government Printing Office* **EPA 600/P-002Fb**, 14.11-14.15.

EPA, U. S. (2000). Benchmark Dose Technical Guidance Document External Review Draft. *Risk Assessment Forum* **http://www.epa.gov/ncea/pdfs/bmds/BMD-External_10_13_2000.pdf**.

Fort, D. J., R. L. Rogers, J. W. Gorsuch, L. T. Navarro, R. Peter and J. R. Plautz (2010). Triclosan and anuran metamorphosis: no effect on thyroid-mediated metamorphosis in Xenopus laevis. *Toxicol Sci* **113**, 392-400.

Friesema, E. C., R. Docter, E. P. Moerings, B. Stieger, B. Hagenbuch, P. J. Meier, E. P. Krenning, G. Hennemann and T. J. Visser (1999). Identification of thyroid hormone transporters. *Biochemical and biophysical research communications* **254**, 497-501.

Gauger, K. J., Y. Kato, K. Haraguchi, H. J. Lehmler, L. W. Robertson, R. Bansal and R. T. Zoeller (2004). Polychlorinated biphenyls (PCBs) exert thyroid hormone-like effects in the fetal rat brain but do not bind to thyroid hormone receptors. *Environmental health perspectives* **112**, 516-523.

Gauger, K. J., S. Giera, D. S. Sharlin, R. Bansal, E. Iannacone and R. T. Zoeller (2007). Polychlorinated biphenyls 105 and 118 form thyroid hormone receptor agonists after cytochrome P4501A1 activation in rat pituitary GH3 cells. *Environmental health perspectives* **115**, 1623-1630.

Gilbert, M. E., W. R. Mundy and K. M. Crofton (2000). Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. *Toxicol Sci* **57**, 102-111.

Gilbert, M. E. (2004). Alterations in synaptic transmission and plasticity in area CA1 of adult hippocampus following developmental hypothyroidism. *Brain Res Dev Brain Res* **148**, 11-18.

Gilbert, M. E. and L. Sui (2006). Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. *Brain Res* **1069**, 10-22.

Goldey, E. S., L. S. Kehn, G. L. Rehnberg and K. M. Crofton (1995). Effects of developmental hypothyroidism on auditory and motor function in the rat. *Toxicology and applied pharmacology* **135**, 67-76.

Goldey, E. S. and K. M. Crofton (1998). Thyroxine replacement attenuates hypothyroxinemia, hearing loss, and motor deficits following developmental exposure to Aroclor 1254 in rats. *Toxicol Sci* **45**, 94-105.

Greenwood, F. C., W. M. Hunter and J. S. Glover (1963). The Preparation of I-131-Labelled Human Growth Hormone of High Specific Radioactivity. *Biochem J* **89**, 114-123.

Haddow, J. E., G. E. Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *The New England journal of medicine* **341**, 549-555.

Hanioka, N., E. Omae, T. Nishimura, H. Jinno, S. Onodera, R. Yoda and M. Ando (1996). Interaction of 2,4,4'-trichloro-2'-hydroxydiphenyl ether with microsomal cytochrome P450-dependent monooxygenases in rat liver. *Chemosphere* **33**, 265-276.

Hanioka, N., H. Jinno, T. Nishimura and M. Ando (1997). Effect of 2,4,4'-trichloro-2'-hydroxydiphenyl ether on cytochrome P450 enzymes in the rat liver. *Chemosphere* **34**, 719-730.

He, X. J., N. Ejiri, H. Nakayama and K. Doi (2005a). Effects of pregnancy on CYPs protein expression in rat liver. *Exp Mol Pathol* **78**, 64-70.

He, X. J., N. Ejiri, H. Nakayama and K. Doi (2005b). Changes in cytochrome P450 isozymes (CYPs) protein levels during lactation in rat liver. *Exp Mol Pathol* **79**, 224-228.

Henrichs, J., J. J. Bongers-Schokking, J. J. Schenk, A. Ghassabian, H. G. Schmidt, T. J. Visser, H. Hooijkaas, S. M. de Muinck Keizer-Schrama, A. Hofman, V. V. Jaddoe, W. Visser, E. A. Steegers, F. C. Verhulst, Y. B. de Rijke and H. Tiemeier (2010). Maternal thyroid function during early pregnancy and cognitive functioning in early childhood: the generation R study. *J Clin Endocrinol Metab* **95**, 4227-4234.

Hood, A. and C. D. Klaassen (2000). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**, 78-84.

Hovander, L., T. Malmberg, M. Athanasiadou, I. Athanassiadis, S. Rahm, A. Bergman and E. K. Wehler (2002). Identification of hydroxylated PCB metabolites and other phenolic halogenated pollutants in human blood plasma. *Archives of environmental contamination and toxicology* **42**, 105-117.

Jigorel, E., M. Le Vee, C. Boursier-Neyret, Y. Parmentier and O. Fardel (2006). Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **34**, 1756-1763.

Jinno, H., N. Hanioka, S. Onodera, T. Nishimura and M. Ando (1997). Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel. *Xenobiotica; the fate of foreign compounds in biological systems* **27**, 681-692.

Johnson, D. R. and C. D. Klaassen (2002). Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. *Toxicol Sci* **67**, 182-189.

Jordan, D., B. Rousset, F. Perrin, M. Fournier and J. Orgiazzi (1980). Evidence for circadian variations in serum thyrotropin, 3,5,3'-triiodothyronine, and thyroxine in the rat. *Endocrinology* **107**, 1245-1248.

Kodavanti, P. R., C. G. Coburn, V. C. Moser, R. C. MacPhail, S. E. Fenton, T. E. Stoker, J. L. Rayner, K. Kannan and L. S. Birnbaum (2010). Developmental exposure to a commercial PBDE mixture, DE-71: neurobehavioral, hormonal, and reproductive effects. *Toxicol Sci* **116**, 297-312.

Kooistra, L., S. Crawford, A. L. van Baar, E. P. Brouwers and V. J. Pop (2006). Neonatal effects of maternal hypothyroxinemia during early pregnancy. *Pediatrics* **117**, 161-167.

Kretschmer, X. C. and W. S. Baldwin (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chemico-biological interactions* **155**, 111-128.

Lavado-Autric, R., E. Auso, J. V. Garcia-Velasco, C. Arufe Mdel, F. Escobar del Rey, P. Berbel and G. Morreale de Escobar (2003). Early maternal hypothyroxinemia alters

histogenesis and cerebral cortex cytoarchitecture of the progeny. *The Journal of clinical investigation* **111**, 1073-1082.

Li, Y., Z. Shan, W. Teng, X. Yu, C. Fan, X. Teng, R. Guo, H. Wang, J. Li, Y. Chen, W. Wang, M. Chawinga, L. Zhang, L. Yang, Y. Zhao and T. Hua (2010). Abnormalities of maternal thyroid function during pregnancy affect neuropsychological development of their children at 25-30 months. *Clinical endocrinology* **72**, 825-829.

Liu, J., Y. Liu, R. A. Barter and C. D. Klaassen (1995). Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *The Journal of pharmacology and experimental therapeutics* **273**, 977-985.

Livak, K. J. and T. D. Schmittgen (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif* **25**, 402-408.

Morse, D. C., D. Groen, M. Veerman, C. J. van Amerongen, H. B. Koeter, A. E. Smits van Prooije, T. J. Visser, J. H. Koeman and A. Brouwer (1993). Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. *Toxicology and applied pharmacology* **122**, 27-33.

Paul, K. B., J. M. Hedge, M. J. Devito and K. M. Crofton (2010a). Developmental triclosan exposure decreases maternal and neonatal thyroxine in rats. *Environmental toxicology and chemistry / SETAC* **29**, 2840-2844.

Paul, K. B., J. M. Hedge, M. J. DeVito and K. M. Crofton (2010b). Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol Sci* **113**, 367-379.

Pohjanvirta, R., M. Niittynen, J. Linden, P. C. Boutros, I. D. Moffat and A. B. Okey (2006). Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin-treated rats. *Chemico-biological interactions* **160**, 134-149.

Pop, V. J., J. L. Kuijpens, A. L. van Baar, G. Verkerk, M. M. van Son, J. J. de Vijlder, T. Vulsma, W. M. Wiersinga, H. A. Drexhage and H. L. Vader (1999). Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clinical endocrinology* **50**, 149-155.

Pop, V. J., E. P. Brouwers, H. L. Vader, T. Vulsma, A. L. van Baar and J. J. de Vijlder (2003). Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clinical endocrinology* **59**, 282-288.

Richardson, T. A. and C. D. Klaassen (2010). Disruption of thyroid hormone homeostasis in Ugt1a-deficient Gunn rats by microsomal enzyme inducers is not due to enhanced thyroxine glucuronidation. *Toxicology and applied pharmacology* **248**, 38-44.

Rodriguez, P. E. and M. S. Sanchez (2010). Maternal exposure to triclosan impairs thyroid homeostasis and female pubertal development in wistar rat offspring. *Journal of toxicology and environmental health* **73**, 1678-1688.

Royland, J. E., J. S. Parker and M. E. Gilbert (2008). A genomic analysis of subclinical hypothyroidism in hippocampus and neocortex of the developing rat brain. *Journal of neuroendocrinology* **20**, 1319-1338.

Sharlin, D. S., R. Bansal and R. T. Zoeller (2006). Polychlorinated biphenyls exert selective effects on cellular composition of white matter in a manner inconsistent with thyroid hormone insufficiency. *Endocrinology* **147**, 846-858.

Sharlin, D. S., D. Tighe, M. E. Gilbert and R. T. Zoeller (2008). The balance between oligodendrocyte and astrocyte production in major white matter tracts is linearly related to serum total thyroxine. *Endocrinology* **149**, 2527-2536.

Sugatani, J., T. Sueyoshi, M. Negishi and M. Miwa (2005). Regulation of the human UGT1A1 gene by nuclear receptors constitutive active/androstane receptor, pregnane X receptor, and glucocorticoid receptor. *Methods in enzymology* **400**, 92-104.

Szabo, D. T., V. M. Richardson, D. G. Ross, J. J. Diliberto, P. R. Kodavanti and L. S. Birnbaum (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression involved in thyroid hormone metabolism in male rat pups. *Toxicol Sci* **107**, 27-39.

Turncliff, R. Z., P. J. Meier and K. L. Brouwer (2004). Effect of dexamethasone treatment on the expression and function of transport proteins in sandwich-cultured rat hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 834-839.

Vansell, N. R. and C. D. Klaassen (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicology and applied pharmacology* **176**, 187-194.

Vansell, N. R. and C. D. Klaassen (2002). Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 240-246.

Vermiglio, F., V. P. Lo Presti, M. Moleti, M. Sidoti, G. Tortorella, G. Scaffidi, M. G. Castagna, F. Mattina, M. A. Violi, A. Crisa, A. Artemisia and F. Trimarchi (2004). Attention deficit and hyperactivity disorders in the offspring of mothers exposed to mild-moderate iodine deficiency: a possible novel iodine deficiency disorder in developed countries. *J Clin Endocrinol Metab* **89**, 6054-6060.

Wang, L. H., A. M. Rudolph and L. Z. Benet (1986). Pharmacokinetic studies of the disposition of acetaminophen in the sheep maternal-placental-fetal unit. *The Journal of pharmacology and experimental therapeutics* **238**, 198-205.

Williams, G. R. (2008). Neurodevelopmental and neurophysiological actions of thyroid hormone. *Journal of neuroendocrinology* **20**, 784-794.

Wolff, M. S., S. L. Teitelbaum, G. Windham, S. M. Pinney, J. A. Britton, C. Chelimo, J. Godbold, F. Biro, L. H. Kushi, C. M. Pfeiffer and A. M. Calafat (2007). Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. *Environmental health perspectives* **115**, 116-121.

Zhou, T., D. G. Ross, M. J. DeVito and K. M. Crofton (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zhou, T., M. M. Taylor, M. J. DeVito and K. M. Crofton (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci* **66**, 105-116.

Zoeller, R. T. and J. Rovet (2004). Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. *Journal of neuroendocrinology* **16**, 809-818.

Zoeller, R. T. and K. M. Crofton (2005). Mode of action: developmental thyroid hormone insufficiency--neurological abnormalities resulting from exposure to propylthiouracil. *Critical reviews in toxicology* **35**, 771-781.

Zoeller, R. T., S. W. Tan and R. W. Tyl (2007). General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Critical reviews in toxicology* **37**, 11-53.

Zorrilla, L. M., E. K. Gibson, S. C. Jeffay, K. M. Crofton, W. R. Setzer, R. L. Cooper and T. E. Stoker (2009). The effects of triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol Sci* **107**, 56-64.

Human relevance of triclosan-induced hypothyroxinemia: Evidence for triclosan activation of human and rodent xenobiotic nuclear receptors

Chapter 5

Katie B. Paul[1], Steve O. Simmons[2], Jack Vanden Heuvel[3], Kevin M. Crofton[2]

[1]University of North Carolina at Chapel Hill, Curriculum in Toxicology, CB 7270, Chapel Hill, NC 27599; [2]Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, RTP, NC 27711; [3]INDIGO BioSciences, State College, PA 16801

1. Abstract

Triclosan (2,4,4'-trichloro-2'-hydroxyphenylether) (TCS) is a chlorinated biphenyl antibacterial found in numerous personal care products, and through use and disposal, in human sera, milk, and urine, and in surface waters. Triclosan (TCS) decreases serum thyroxine (T4) in the rat. Previous research has focused on the *in vivo* mode-of-action (MOA) of TCS-induced hypothyroxinemia observed in rats; results suggested that TCS up-regulates hepatic catabolism of thyroid hormones, resulting in increased clearance of thyroid hormones. Key biomarkers of Cyp2b2, Cyp3a1, Ugt1a1, Sult1c1, and glucuronidation of T4 were up-regulated. The expression of these Phase I and Phase II enzymes is regulated by nuclear receptors including the constitutive androstane receptor (CAR) and pregnane-X receptor (PXR), but evolutionary divergence of these receptors has made activation of these receptors a species-dependent event, indicating the possibility that downstream up-regulation of hepatic catabolism may also be a species-dependent event. To test the hypothesis that TCS may activate xenobiotic nuclear receptors in both rats and humans, and that the increased biomarkers of hepatic catabolism and decreased thyroid hormones observed in rats may be possible in humans, cell-based nuclear receptor reporter assays were employed to indirectly measure the ability of TCS to activate human (hPXR) and rat PXR (rPXR), all three biologically-active splice variants of human CAR (hCAR), and rat CAR (rCAR). Additional data was also collected on mouse CAR (mCAR) and PXR (mPXR) for comparison. TCS moderately activated human PXR in both full-length and chimeric receptor-reporter assays by approximately 2- to 3-fold vehicle control at about 10 µM ; strongly activated hCAR2 (19.8 ± 4.9-fold vehicle control 30 µM TCS); and, was a moderate agonist of hCAR3 (2.63 ± 0.75-fold vehicle control 30 µM TCS). In addition, TCS acted as

an inverse agonist of hCAR1(EC50 = 19.8 µM). TCS failed to activate rPXR in both receptor-reporter models, and instead acted as a modest inverse agonist of rCAR (EC50 = 3.50 µM TCS). TCS failed to activate mouse PXR and was an inverse agonist of mouse CAR, which reflected the data for the rat receptors. These data suggest that TCS may be capable of interacting with rat CAR, activating human CAR and PXR in order to potentially up-regulate hepatic catabolism in both species. Although TCs activated different nuclear receptors between species, activation of nuclear receptors in both species is likely to result in up-regulation of hepatic catabolism. This is consistent with *in vivo* observations in the rat. Overall these data support the conclusion that TCS may have similar adverse effects in all three species assuming doses are high enough to activate hepatic xenobiotic receptors.

2. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxyphenylether) decreases thyroxine (T4) in juvenile rats (Crofton *et al.*, 2007; Paul *et al.*, 2010b; Zorrilla *et al.*, 2009) as well as maternal, fetal, and early neonatal thyroxine following developmental exposures (Paul *et al.*, 2010a). Decreased maternal T4 during gestation leads to irreversible decreases in neurodevelopment and motor function in humans (Haddow *et al.*, 1999; Pop *et al.*, 2003; Pop *et al.*, 1999; Vermiglio *et al.*, 2004), and neurological deficits and alterations in the cellular organization of the brain in rats (Auso *et al.*, 2004; Gilbert *et al.*, 2000; Gilbert *et al.*, 2006; Goldey *et al.*, 1998; Goldey *et al.*, 1995; Lavado-Autric *et al.*, 2003; Opazo *et al.*, 2008). Although the general mechanisms for thyroid hormone maintenance and biology are conserved across species (Yen, 2001; Zoeller *et al.*, 2007), interspecies-differences can present a major uncertainty when using solely *in vivo* animal data for human health risk

assessment. Determination of a proposed MOA in rats enables parallel hypotheses to be investigated for plausibility in humans using *in vitro* models (Boobis *et al.*, 2008).

Thyroid-disrupting chemicals may act via one or more of several proposed MOAs, including by inhibition of iodine uptake or enzymatic synthesis of thyroid hormones in the thyroid gland; by inhibition of peripheral deiodination of T4 to triiodothyronine (T3); by displacement of thyroid hormones from serum binding proteins in the systemic circulation; and by up-regulation of hepatic catabolism and transport of thyroid hormones, resulting in increased, disproportionate excretion of thyroid hormones (Boas *et al.*, 2006; Capen, 1994; Crofton, 2008; Miller *et al.*, 2009). The hypothyroxinemia induced by TCS exposure in rats as been hypothesized to be the result of up-regulation of hepatic catabolism of thyroid hormones. *In vitro* treatment of rat hepatocytes resulted in increased Cyp2b and Cyp3a1 protein and enzymatic activity increases in markers of cytochrome P450 activity including pentoxyresorufin-O-depentylase (PROD), benzyloxyresorufin-O-debentylase (BROD), and ethoxyresorufin-O-deethylase (EROD) (Jinno *et al.*, 1997). *In vivo* triclosan treatment of rats also induced hepatic Cyp2b, Cyp3a, and Cyp4a protein and PROD and BROD activity (Hanioka *et al.*, 1997). Evidence of Phase I induction *in vitro* and *in vivo*, in addition to *in vitro* pregnane X receptor (PXR) activation by TCS (Jacobs *et al.*, 2005), suggest that TCS activates nuclear receptors, in particular the constitutive androstane receptor (CAR) and/or PXR, as indicated by inductive effects on the cytochrome P450s primarily regulated by these receptors, Cyp2b and Cyp3a respectively (Goodwin *et al.*, 2001; Maglich *et al.*, 2002; Moore *et al.*, 2002). Our previous work with TCS in a four-day exposure to young adult rats demonstrated that TCS up-regulated cytochrome P450 mRNA expression and activity and Phase II hepatic catabolism; specifically, hepatic mRNA expression levels of *Cyp2b2*,

Cyp3a1/23, *Ugt1a1*, and *Sult1c1*, as well as hepatic microsomal uridine diphosphate glucuronyltransferase (UGT) activity and pentoxyresorufin-O-deethylase (PROD) activity, were induced by triclosan treatment (Paul et al., 2010b). These effects are consistent with other microsomal enzyme inducers that are thought to decrease thyroid hormones at least partially via up-regulation of hepatic catabolism (Barter *et al.*, 1994; Chen *et al.*, 2003; Hood *et al.*, 2003; Vansell *et al.*, 2002; Vansell *et al.*, 2001). In aggregate the data suggest that TCS may activate CAR and/or PXR in the rat, and PXR in humans.

CAR and PXR function at the nexus of exogenous and endogenous biology, regulating expression of cytochrome P450s, UDP-glucuronyltransferases (UGTs), sulfotransferases (SULTs), and drug transporters to mount metabolic responses to xenobiotics (Goodwin et al., 2001; Guo *et al.*, 2003; Lehmann *et al.*, 1998; Maglich et al., 2002; Moore et al., 2002; Wei *et al.*, 2002; Zhou *et al.*, 2005), but also to regulate hepatic energy metabolism (Konno *et al.*, 2008; Maglich *et al.*, 2009; Maglich *et al.*, 2003; Maglich *et al.*, 2004), detoxify and excrete bilirubin, and prevent cholestatic injury by increasing elimination of bile acids (Guo et al., 2003; Staudinger *et al.*, 2001; Zhang *et al.*, 2004). Upon activation by xenobiotics, CAR and PXR heterodimerize with the retinoid-X receptor (RXRα), recruit co-activators and/or release co-repressors, and bind to promoter response elements to transcriptionally regulate an overlapping set of genes necessary for maintenance of euthyroid status (Kretschmer *et al.*, 2005), as well as elimination of xenobiotics and endogenous energy and lipid metabolism. Generally PXR and CAR are the primary regulators of Cyp3a and Cyp2b domains (Wang *et al.*, 2003c), respectively, though the precise isoform is species-dependent and there is considerable cross-talk between receptors due to competition for an overlapping set of promoter response elements (Faucette *et al.*,

2006; Goodwin et al., 2001; Istrate *et al.*, 2010; Smirlis *et al.*, 2001; Wei et al., 2002). Activation of human PXR (hPXR) results in transcriptional induction of CYP3A4 (Jones *et al.*, 2000), as well as other Phase I and II hepatic biotransformation enzymes, i.e. glucuronyltransferases and sulfotransferases that catabolize thyroid hormones as part of thyroid hormone homeostasis (Capen, 1994; DeVito *et al.*, 1999; Schuur *et al.*, 1997; Wang *et al.*, 2006), and Phase III transporters, including organic anion transporting peptides and multidrug resistance proteins (Burk *et al.*, 2005; Geick *et al.*, 2001; Kretschmer et al., 2005), which are known to mediate hepatic uptake and secretion of thyroid hormones (Jansen *et al.*, 2005). Similarly, activation of human CAR results in transcriptional up-regulation of CYP2B6 (Maglich et al., 2002; Sueyoshi *et al.*, 1999; Wang *et al.*, 2004; Wang *et al.*, 2003a), as well as UGT1A1, SULT2A1, and hepatic transporters including multi-drug resistance associated proteins MRP2 and MRP3 and the multi-drug resistance protein MDR1 (Urquhart *et al.*, 2007). Murine knockout models have demonstrated that CAR and PXR are necessary for the downstream effects of compounds including phenobarbital (Qatani et al 2005) and PCN (Chen et al., 2003; Cheng *et al.*, 2006), respectively, on glucuronidation and thyroid hormone elimination. Increased expression and activity of the Ugt1a family of glucuronyltransferases that conjugate thyroid hormones (Barter et al., 1994; Liu *et al.*, 1995) is well established for microsomal enzyme inducers that activate CAR and PXR (Buckley *et al.*, 2009). Activation of CAR and/or PXR is the putative initiating key event that may result in up-regulation of metabolic enzymes that catabolize and transport T4, potentially decreasing serum concentrations of T4 via metabolic turnover.

Activation of CAR and PXR by xenobiotics is exceedingly species-dependent. Two contributing factors, among myriad potential biological differences in the activation of these

receptors, to this species-dependent activation are: (1) the divergence of the ligand-binding domains of the receptors across species; and, (2) differential expression of splice variants of these receptors by species. PXR ligand-binding domains (LBDs) have diverged considerably through evolution, such that the LBD of rat and mouse PXR 75-77% homologous to the LBD of human CAR (Jones et al., 2000; Moore et al., 2002). The DNA-binding domains (DBDs) remain highly conserved with over 95% homology between murine and human PXRs (Iyer *et al.*, 2006; Jones et al., 2000; Timsit *et al.*, 2007). The variation in the LBD across species poses a major uncertainty for extrapolating from murine models to humans. A significant difference between murine CAR and human CAR is the variation in the transcriptome: rat CAR and mouse CAR have only one known functional splice variant each, and hCAR has three active splice variants. These three splice variants of human CAR include hCAR1 or wildtype, hCAR2, and hCAR3 (Auerbach *et al.*, 2003; Jinno *et al.*, 2004; Savkur *et al.*, 2003) comprising the transcriptome for hCAR. The hCAR1 variant compromises the majority of hCAR expression in the liver. Recent reports suggest that hCAR2 may comprise up to 30% of the transcriptome, and further that hCAR2 and hCAR3 together may comprise up to 50% of the transcriptome and possess the majority of the ligand-binding activity demonstrated by the transcriptome (DeKeyser *et al.*, 2009; Ross *et al.*, 2010; Savkur et al., 2003). All three hCAR SVs bind response elements from promoters of CYP2B6 and CYP3A4 (Auerbach *et al.*, 2007; Auerbach *et al.*, 2005). The reference form hCAR1 constitutively activates the PBREM and XREM response elements in the CYP2B6 and CYP3A4 promoter regions, unlike hCAR2 and hCAR3, which appear to be highly ligand-dependent for nuclear translocation (DeKeyser et al., 2009). In contrast, hCAR2 is ligand-dependent and hCAR3 is somewhat ligand-dependent for activation due to amino acid insertions in the ligand-binding

domain (Auerbach et al., 2007; Auerbach et al., 2003). Thus, there is certainly the potential for different hCAR splice variants to bind agonists with varying affinity, or different agonists/activators altogether. The result of the interplay of these three SVs in response to an agonist or activator remains unclear from nuclear receptor-reporter assays, and requires analysis of the downstream metabolic responses of primary hepatocytes or whole animals that contain the entire hCAR transcriptome. Due to the clearly different roles for the three human CAR SVs, it is difficult to compare rat and mouse CAR to human CAR. Together the differences between murine CAR and PXR and human CAR and PXR necessitate assessment of receptors across species to determine if a chemical such as TCS can activate receptors in both murine and human models.

There are numerous examples of species-dependent activation of CAR and PXR by xenobiotics that are also known to perturb thyroid hormones. The compounds 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO), carbamazepine, efavirenz, and nevirapine are known to preferentially activate human CAR and not mouse nor rat CAR (Chang *et al.*, 2006; Faucette *et al.*, 2007; Moore *et al.*, 2000). Conversely,1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is a known murine CAR activator (Buckley et al., 2009; Maglich et al., 2009; Tzameli *et al.*, 2000). Rifampicin, perhaps the most recognized for its species-selectivity, and hyperforin of St. John's Wort, selectively activate human PXR (Jones et al., 2000; LeCluyse, 2001; Lehmann et al., 1998; Sinz *et al.*, 2007; Watkins et al., 2003), and pregnenolone-16α-carbonitrile (PCN) is generally recognized as a selective murine agonist of PXR (Cheng et al., 2006; Cheng *et al.*, 2005; Guo *et al.*, 2002a; Guo *et al.*, 2002b; Sinz et al., 2007). However, there are also chemicals that can activate both human and murine receptors, including TO-901317

for PXR (Mitro *et al.*, 2007) and DEHP (DeKeyser et al., 2009; Ren *et al.*, 2010) aphenobarbital for CAR (Mutoh *et al.*, 2009; Qatanani *et al.*, 2005a; Sueyoshi *et al.*, 2001; Yueh *et al.*, 2005). It is important to underscore that inducers of hCAR transcriptional activity may be divided into two categories: direct agonists and indirect activators of CAR. Treatment with phenobarbital, an indirect activator of CAR, results in protein kinase C-mediated dephosphorylation of threonine 38 of hCAR, with subsequent translocation and transcriptional activity (Mutoh *et al.*, 2009), and the ligand-independent reference form hCAR1 has been reported to be responsive to phenobarbital (Jinno et al., 2004). Phenobarbital presents a prime example of the difficulty in comparing human and rodent receptors: if mouse and rat only have one CAR splice variant each, how does this splice variant potentially function through the ligand-dependent and ligand-independent mechanisms suggested by the activity of human CAR splice variants? There is a demonstrable need to examine a chemical like TCS across species to determine if the receptor-regulated downstream biology observed in the rat is plausible for humans.

The present work tested the hypothesis that the *in vivo* effects of TCS in rats on hepatic catabolism and thyroid hormones were mediated by interaction with rat PXR and/or rat CAR, and, further, that TCS could also activate human PXR and/or CAR in order to mediate a similar cascade of hepatic catabolic events in humans. Two distinct types of receptor-reporter assays were employed to test this hypothesis. First, cell-based full-length receptor reporter assays (Puracyp, Inc.) were used to determine if TCS is an agonist for hPXR and rPXR and to measure induction of CYP3A activity *in vitro*. Second, chimeric receptor constructs, with the native DNA-binding domain replaced by the yeast transcription factor Gal4 (INDIGO BioSciences), were used to determine if TCS could interact with the

three splice variants of human CAR (hCAR1, hCAR2, hCAR3) and rCAR, and to confirm our original findings with the full-length hPXR and rPXR cell lines. Additionally, these chimeric receptor reporter assays were used to analyze the potential interaction for TCS with mCAR and mPXR. Collectively these data address a major uncertainty regarding the potential interspecies response to TCS at the molecular level of interaction with species-divergent nuclear receptors, and the capacity for TCS to initiate a proposed MOA for TCS-induced hypothyroxinemia in humans.

3. Methods

a. Chemicals and treatment. Rifampicin (≥97%) and dexamethasone (≥97%) were obtained from Sigma (St. Louis, MO) and triclosan (≥99%) was a gift from BASF/Ciba Specialty Chemicals. Dimethyl sulfoxide (DMSO) (Sigma, ≥99.7%) was the vehicle solvent for all chemicals used. Chemicals were administered to 96-well plates of cells using a BioMek 2000 (Beckman Coulter; Brea, CA), which robotically transferred 327 nanoliters of chemical solution to each treated well.

b. Full-length receptor reporter assays (Puracyp, Inc.). A HepG2 cell line (DPX2) with a stable transfection of the full length human PXR and PXR response elements within the CYP3A4 promoter was obtained from Puracyp, Inc. (Carlsbad, CA). A FAO cell line (RPXR) containing the full length rat PXR and PXR response elements within the CYP3A4 promoter was also obtained from Puracyp, Inc. (Carlsbad, CA). Construction and subsequent validation of these stably-transfected cell lines has been reported previously (Raucy et al 2002, 2003, Yueh et al 2007). The cells were maintained per the company's directions for a maximum of 30 days in culture (10-12 passages) to ensure optimal activity in luciferase induction assays. For experimental analysis of luciferase induction, cells were harvested

from 10 cm dishes at 50-70% confluency, and re-seeded in 96-well plates (2×10^4 cells/well and 1×10^4 cells/well in 165 µL of media for the DPX2 and RPXR cells, respectively), and allowed to recover overnight. Each chemical concentration was tested in technical triplicates on three separate occasions for n=3; each of the three separate experiments was performed using cells from the same lot, but a consecutive cell passage. Identical plates of treated cells were performed in parallel for separate assessment of luciferase induction and cytotoxicity. TCS exposure concentrations ranged from 0.387 to 99.1 µM; rifampicin exposure concentrations ranged from 0.0968 to 24.8 µM; and, dexamethasone exposure concentrations ranged from 0.387 to 99.1 µM. Using the molecular weight of TCS (289.54 g/mol), the *in vitro* TCS concentration range can be expressed as 0.112 to 28.7 mg/L. The *in vivo* doses used in Chapters 2, 3, and 4 of this dissertation work, 10 to 1000 mg/kg/day, can similarly be converted to 34 to 3400 µmol/kg/day. Following chemical treatment, cells were exposed for 24 hr prior to measurement of luciferase induction (for PXR or CYP3A activity) and cytotoxicity. For the time-course experiments, cells were incubated for 6, 12, 24 or 48 hr before analysis of luciferase induction and cytotoxicity. Luciferase induction was measured using BrightGlo luciferase detection reagent (Promega; Madison, WI) and cytotoxicity was measured using CellTiterGlo (Promega; Madison, WI). Signal detection of luciferase for both the induction and cytotoxicity assays was performed with a FLUOstar Optima BMG plate reader (BMG LabTech, Offenburg, Germany).

c. CYP3A activity assays in Puracyp receptor-reporter cell lines. CYP3A activity was measured in RPXR and DPX2 cells following a 24 hr incubation with the test chemicals. The P450-Glo™ CYP3A4 assay with luciferin-IPA (Promega; Madison, WI) was conducted per the manufacturer's directions. Briefly, media from well of a 96-well plate was

aspirated and replaced with 30 uL of diluted (1:1000) luciferin-IPA reagent. The plate was then incubated at 37°C for 30 min before addition of 30 uL of the reconstituted luciferase detection reagent. The plate was allowed to equilibrate to room termperature for 20 min before signal detection of luciferase activity with a FLUOstar Optima BMG plate reader (BMG LabTech, Offenburg, Germany).

d. Chimeric receptor-reporter assays (INDIGO Biosciences). HEK293T fibroblasts were

transiently transfected with a chimeric receptor construct where the DNA-binding domain for the receptors has been replaced with a GAL4 element under regulatory control of the SV40 promoter. Cells were co-transfected with a reporter plasmid containing the fusion of the UAS GAL4 DNA response element to the firefly luciferase gene along with a transfection efficiency control vector containing a renilla luciferase reporter element (pRL-luciferase; Promega; Madison, WI) used to determine viable, transfected cell fraction. Use and validation of these cell lines has been previously reported (Tien *et al.*, 2006; Vanden Heuvel *et al.*, 2006). The luciferase-based reporter assays were conducted using the Luciferase dual reporter assay kit (Promega; Madison, WI) and a Tecan GeniosPro luminescent plate reader (Research Triangle Park, NC).

e. Data analysis.

i. Puracyp cell lines. All results obtained for PXR activation and CYP3A4 induction were in relative luminescence units (RLU). To correct for differences in cell viability following chemical exposure, the RLU was divided by the corresponding RLU for cell viability, as measured in a parallel plate. This ratio was then converted to a percent of DMSO vehicle control for the time-course data and CYP3A4 activation data, and to a percent

of maximal positive control response for PXR activation at 24 hr. These computations were performed with SAS 9.2 (SAS Institute; Cary, NC). Four parameter logistic models were used to determine EC50s for positive control data using 6 to 10 concentrations of each chemical (SigmaPlot 11.0, GmbH, Germany).

ii. INDIGO cell lines. All results obtained for receptor activation were also in RLU, which were corrected by Renilla luciferase units that represented cell viability. The RLU/renilla luciferase ratio was converted to a percent of DMSO vehicle control (SAS 9.2, SAS Institute; Cary, NC). Due to the limited number of data points for many of the chemicals tested, data were analyzed by ANOVAs to determine effects significantly different from the DMSO vehicle control, with significant main effects of treatment followed by Duncan's new multiple range test. For those chemicals where a full dose-response curve could be achieved, with a minimum and maximum, a four parameter logistic model was used to determine EC50s (SigmaPlot 11.0, GmbH, Germany).

4. Results

a. Full-length hPXR and rPXR reporter assays

Rifampicin (RIF) activated the full-length hPXR with maximal efficacy at 24.8 µM at all time points tested; activation was best detected at 24 and 48 hr of chemical exposure, and produced 1671 ± 91.4 and 3722 ± 597 percent of vehicle control RLU, respectively (Figure 5.1A). The EC50s for RIF at 24 and 48 hr were 1.07 ± 0.060 and 1.20 ± 0.086 µM, respectively (Figure 5.1A). TCS activated the full-length hPXR with maximal efficacy at 24.8 µM with the best detection at 24 and 48 hr, which produced 1165 ± 158 and 2199 ± 352 percent of vehicle control RLU, respectively (Figure 5.1B). The dose-response curve for TCS activation of hPXR is truncated at 24.8 µM due to overwhelming cytotoxicity in DPX-2

cells at 49.5 µM and above. Therefore, an EC50 using a Hill model cannot be computed because a maximal efficacy cannot be achieved without considerable cytotoxicity, and subsequent analysis of TCS-induced activation of the full-length hPXR is reported as a percent of maximal RIF response. Following a 24 hr treatment period, TCS activated the full-length hPXR by 12.3% ± 1.2, 25.3% ± 1.3, and 64.4% ± 7.8 of the maximal RIF response at 6.2, 12.4, and 24.8 µM TCS, where the maximal efficacy of RIF was 100% ± 2.48 at 12.4 µM in DPX-2 cells (Figure 5.3A). Since a maximum efficacy could not be obtained with TCS exposure due to cytotoxicity, a rough comparison of the estimated TCS concentration (20 µM) that corresponds to the RIF EC50 concentration (1 µM) demonstrates an approximate 20-fold difference in potency in the full-length hPXR assay between TCS and RIF.

Dexamethasone (DEX) activated full-length rPXR with maximal efficacy at 49.5 µM at all time points tested, and again detection was optimal at 24 and 48 hr of chemical exposure (Figure 5.2A). The EC50s for DEX at 24 and 48 hr were 3.21 ± 0.22 and 3.93 ± 0.42 µM, respectively (Figure 5.2A). TCS failed to activate the full-length rPXR at any dose in the RPXR cells. The TCS dose-response curve was truncated at 12.4 µM as fewer than 50% of RPXR cells were viable at higher concentrations (Figure 2B). Following a 24 hr incubation, DEX achieved maximal efficacy at 49.5 µM (100.6% ± 4.7) in RPXR cells (Figure 5.3B). No relative TCS potency can be reported due to a lack of TCS activity in this full-length rPXR reporter assay.

CYP3A4 induction by RIF and TCS in DPX-2 cells mirrored the PXR activation response observed with the full-length hPXR model (Figure 5.4A). Maximal efficacy was obtained once again with 12.4 µM RIF to produce an effect that was 866 ± 44 percent of

DMSO vehicle control (Figure 5.4A). Exposure to 12.4 and 24.8 µM TCS produced a response of 178 ± 5.4 and 288 ± 16 percent of DMSO vehicle control, respectively (Figure 5.4A). A relative potency estimate between the approximate RIF EC30 (0.9 µM) and the corresponding TCS concentration that induced the same CYP3A4 activity (24.8 µM) illustrates about a 30-fold potency difference between RIF and TCS for CYP3A4 activity induction. Maximal CYP3A4 induction in RPXR cells was obtained with 49.5 µM DEX, which produced a response that 2023 ± 296 percent of DMSO vehicle control (Figure 5.4B). TCS did not induce CYP3A4 in the RPXR cell model at any concentration (Figure 5.4B).

Increasing the length of TCS exposure duration did not appear to affect the resultant cytotoxicity response of DPX-2 and RPXR cells (data not shown; see Appendix 5 for cytotoxicity data over time). With 12.4 µM TCS, there was a decrease of 10-20% in DPX-2 cell viability at all of the time-points tested. With 24.8 µM TCS, there was a decrease of approximately 50% in DPX-2 cell viability at all of the time-points tested. At 49.5 µM TCS, approximately 10% of cells were still viable; thus, all TCS dose-response curves in DPX-2 are truncated at 24.8 µM. In comparison, RPXR cell viability was decreased by approximately 10%, 15-25%, 30-45%, 70-75%, and 100% at 3.10. 6.20, 12.4, 24.8, and 49.5 µM, respectively. TCS initiates cytotoxicity at 6.20 µM in the RPXR cells, a dose tolerated well by DPX-2 cells, and which elicited an approximate 200% of control PXR activation response. This comparison highlights a narrow window between loss of cell viability and activation of hPXR in DPX-2 cells. The concentration that produced the maximal hPXR activation in DPX-2 cells, 24.8 µM, results in 70-75% cell death in RPXR cells.

b. Chimeric receptor reporter assays

There was modest cytotoxicity demonstrated by a 30-40% loss in cell viability with TCS at the maximum concentration of 30 µM TCS in the HEK293T fibroblast cell lines with the chimeric receptor reporters. As the same cell line was used for all of the chimeric receptor reporters, the moderate cytotoxic effects were consistent assay to assay.

TCS significantly activated the chimeric hPXR reporter by 2.41 ± 0.28, 2.84 ± 0.30, and 5.67 ± 0.035-fold vehicle control at 10, 20, and 30 µM TCS [$F(5,11)=39.00$, $p<0.0001$] (Figure 5.5A). The positive control for the chimeric hPXR reporter assay was the known hPXR agonist TO-901317, which activated hPXR up to 7.21 ± 0.13-fold vehicle control at 0.4 µM TO-901317 [$F(5,15)=139.09$, $p<0.0001$] (Figure 5.5A). A comparison of the potency between TO-901317 and TCS, i.e. the TCS concentration that corresponds to the visually estimated EC50 for TO-901317, demonstrates a

TCS failed to activate the chimeric mPXR reporter at any concentration [$F(6,17)=1.45$, $p<0.2549$] (Figure 5.5B). TO-901317 activated the chimeric mPXR reporter 1.67 ± 0.13-fold vehicle control at 2 µM TO-901317 [$F(5,15)=3.48$, $p<0.0274$] (Figure 5.5B).

TCS demonstrated splice-variant dependent activities for the chimeric hCAR reporter assays (Figure 5.6). TCS decreased the activation of hCAR1 to a minimum of 0.28 ± 0.092-fold vehicle control at 30 µM TCS, with an EC50 of 19.8 µM (Figure 5.6A). The positive controls clotrimazole (CTZ) and di-(2-ethylhexyl)phthalate (DEHP) decreased activation of hCAR1, with EC50s of 6.33 and 8.48 µM for CTZ and DEHP, respectively (Figure 5.6A). The control CITCO did not have activity at hCAR1 (Figure 5.6B).

For hCAR2, a ligand-dependent splice variant, TCS acted as a strong agonist of the chimeric receptor (Figure 5.6C) [$F(6, 14)=13.40$, $p<0.0001$]. TCS activated hCAR2 by 4.12

± 0.53, 5.19 ± 0.95, and 19.8 ± 4.9-fold vehicle control at 10, 20, and 30 µM TCS. Previously, DEHP activated hCAR2 greater than 5-fold vehicle control in receptor-reporter assays in HepG2 cells (DeKeyser et al., 2009). CTZ appeared to increase activation of hCAR2, but these results were not dose-dependent and represented a two to three-fold vehicle increase for all concentrations. No effects of CITCO or PB exposure were observed on hCAR2 activation.

For hCAR3, TCS, CITCO, DEHP, and phenobarbital (PB) all demonstrated activation of the chimeric receptor (Figure 6D-E). TCS activated hCAR3 by 3.28 ± 0.41 and 4.14 ± 1.2-fold vehicle control at 20 and 30 µM TCS [$F(6,13)=3.83$, $p<0.0202$] (Figure 6D). DEHP activated hCAR3 up to 3.20 ± 0.61-fold vehicle control [$F(3,8)=5.74$, $p<0.0215$] at 10 µM DEHP and PB activated hCAR3 up to 3.38 ± 0.31-fold control [$F(3,8)=5.66$, $p<0.0223$]. CITCO, the most potent and efficacious agonist, activated hCAR3 by 22.6 ± 0.67 and 66.2 ± 6.4-fold vehicle control at 0.5 and 5 µM CITCO, respectively [$F(3,8)=87.84$, $p<0.0001$] (Figure 6E).

TCS decreased the activation the chimeric rCAR and mCAR. For rCAR, TCS decreased the activation 40-50% of vehicle control at 10-30 µM TCS concentrations, with an EC50 of 3.50 µM TCS [$F(6,14)=20.81$, $p<0.0001$] (Figure 5.7A). The controls TCPOBOP and CTZ also decreased the activation of the chimeric rCAR (Figure 5.7A); TCPOBOP decreased rCAR activation to 0.70 ± 0.033-fold vehicle control at 10 µM TCPOBOP [$F(4,10)=20.37$, $p<0.0001$] with an EC50 of 8.56 µM TCPOBOP, and CTZ decreased rCAR activation to 0.40 ± 0.036-fold control at 12.5 µM CTZ [$F(5,12)=117.09$, $p<0.0001$] with an EC50 of 4.80 µM CTZ. For mCAR, TCS decreased the activation of the chimeric receptor 0.16 to 0.44-fold control for 10-30 µM TCS [$F(6,14)=7.41$, $p<0.0010$] (EC50 computation

failed). PB also decreased the activation of the chimeric mCAR, by 0.43 to 0.76-fold vehicle control for all PB concentrations (0.1 – 100 µM) [$F(4,10)=8.37$, $p<0.0031$] (EC50 computation failed). Conversely, TCPOBOP and CTZ were agonists of the chimeric mCAR, and activated the receptor up to 6.39 ± 0.20 and 8.20 ± 0.33-fold vehicle control for 10 µM TCPOBOP [$F(4,10)=186.50$, $p<0.0001$] and 12.5 µM CTZ [$F(5,12)=201.80$, $p<0.0001$], respectively.

5. Discussion

The current work tested the hypothesis that TCS activates rodent and human CAR and/or PXR, to potentially initiate an up-regulation of hepatic catabolism across multiple species. Consistent with this hypothesis, TCS activated human PXR and had activity at multiple human CAR splice variants. TCS was an agonist for hPXR in both full-length and chimeric receptor reporter assays; and agonist for hCAR2; an agonist of hCAR3; and, an inverse agonist for the constitutively activated hCAR1. TCS failed to activate full-length rPXR and chimeric mPXR; rather, TCS decreased the activation of both rCAR and mCAR suggesting that TCS is an inverse agonist of the murine CAR receptors, similar to the activity of TCS at hCAR1. The data obtained suggest that TCS is capable, given a high enough concentration, of up-regulating hepatic catabolism in humans, including cytochrome P450, glucuronidation and sulfation activity. Intriguingly, these data indicate that TCS may be able to activate CAR in rats and both CAR and PXR in humans, and initiate an up-regulation of hepatic catabolism, though perhaps by an initial nuclear receptor interaction that is species-dependent.

The positive controls RIF and DEX activated hPXR and rPXR in full-length receptor model cell lines, respectively, with EC50 values that are consistent with previous reports

utilizing these commercially-available cell lines (Sinz *et al.*, 2007; Yueh *et al.*, 2005). TO-901317, the positive control for human and mPXR used in the chimeric receptor reporter assays, also obtained similar activation of these receptors to previously reported findings (Mitro et al., 2007). CITCO, CTZ, DEHP, PB, and TCPOBOP activities for the human, rat, and mouse chimeric CARs were also consistent with previous literature reports (Buckley et al., 2009; Chang et al., 2006; Dekeyser *et al.*, 2011; DeKeyser et al., 2009; Faucette et al., 2007; Maglich et al., 2009; Moore et al., 2000; Mutoh et al., 2009; Qatanani *et al.*, 2005b; Sueyoshi et al., 2001; Yueh et al., 2005).

TCS activated hPXR in both the full-length and chimeric receptor reporter assays, indicating that TCS may interact with hPXR to mediate downstream transcriptional up-regulation of Phase I-Phase III metabolic enzymes in humans. TCS was a moderate agonist in both models of hPXR and also increased CYP3A4 activity in the full-length receptor reporter cell lines at 10 to 30 µM TCS. These results confirm a previous report using a luciferase-based human PXR reporter assay in human hepatoma cells that demonstrated moderate *in vitro* activation of PXR by triclosan, as 10 µM triclosan produced 46% of the response of 10 µM of the prototypical inducer rifampicin (Jacobs et al., 2005). This lent support to the hypothesis that the up-regulation of Phase I-II biology observed *in vivo* in the rat is mediated via PXR in a human model. In contrast, we were unable to demonstrate activation of rPXR in either the full-length receptor reporter model of the chimeric receptor reporter model, in direct opposition to our hypothesis, previously published *in vivo* data that demonstrated increased *Cyp3a1* expression (Paul et al., 2010b), and increased Cyp3a1 protein content (Hanioka et al., 1997). However, it is possible that the effects observed on Cyp3a in *in vivo* rodent studies could be the result of CAR activation in the rat.

TCS demonstrated an interesting pattern of effects on hCAR that were splice variant-dependent, and suggest that TCS may also interact with hCAR to potentially up-regulate hepatic catabolism downstream in humans. TCS decreased activation of hCAR1, was a potent agonist of hCAR2, and was a moderate agonist of hCAR3 in the chimeric receptor reporter assays. The effect of TCS on hCAR1 activation is consistent with activity as an inverse agonist of CAR (Baldwin *et al.*, 2008; Chen *et al.*, 2010). Despite seemingly opposed receptor activities, inverse agonism of hCAR1 and agonism of hCAR3 is consistent with other compounds including CITCO and PB (Chen *et al.*, 2010). The unique properties of the three known active splice variants of hCAR create a hypothetical scenario: TCS decreases the high, ligand-independent constitutive activity of hCAR1 and increases the ligand-dependent activity of hCAR2 and hCAR3, which have much lower constitutive activation (Auerbach et al., 2007; Chen *et al.*, 2010). This could result in an increased translocation of activated CAR complex to the nucleus of the cell and transcriptional up-regulation of hCAR target genes such as CYP2B6. Most striking was the observation that TCS is a potent agonist for hCAR2, a highly selective ligand-dependent splice variant of hCAR (Auerbach *et al.*, 2007), with only one other known agonist, DEHP (DeKeyser *et al.*, 2009). These data suggest that both hPXR and hCAR may both be involved in mediating transcriptional responses to TCS exposure.

The results of the rCAR and mCAR assays were similar to the activity displayed by TCS toward hCAR1, with TCS appearing to act as an inverse agonist. Though initially appearing counter to our hypothesis, this activity is mirrored by PB in this model: phenobarbital is an inverse agonist of rCAR, despite well-defined effects on Cyp2b expression that are known to be dependent upon CAR function (Qatanani *et al.*, 2005b; Smirlis et al., 2001; Sueyoshi et

al., 1999). This conundrum may also stem from the complex biology of the rodent CAR; if rCAR and mCAR act like hCAR1 and express high constitutive activation, then it may be difficult to detect and identify ligands/activators for the receptor *in vitro* (Baldwin et al., 2008). Although we were unable to demonstrate activation of rat PXR by TCS in either the chimeric or full-length receptor reporter models, TCS appeared to follow the pattern of PB action, displaying inverse agonism in the rat and mouse CAR assays. The finding that TCS activated hPXR and not rPXR was consistent with previous reports that demonstrate that PB moderately activated hPXR (Fery *et al.*, 2010; Luo *et al.*, 2002) but not rPXR in receptor reporter assays (Fery et al., 2010; Sinz *et al.*, 2007). Based on the similar response of TCS to known microsomal enzyme inducers PB, TCPOBOP, and CTZ in the rCAR assay, we propose that TCS initiates an up-regulation of hepatic catabolism via indirect activation of rCAR in rats.

The data herein suggest several alternative hypotheses due to the unexpected lack of activation of rPXR. First, that only rCAR is responsible for transcriptional up-regulation of the downstream effects observed in rats *in* vivo; second, that the methodology employed is inadequate to model the potential interaction of TCS with rat nuclear receptors *in vivo*; or, third, that other nuclear receptors or factors, in addition to or instead of rCAR and rPXR, contribute to the downstream biology observed *in vivo* in the rat. While rCAR may be the primary driver of the downstream biology observed *in vivo* in the rat, it is not clear if TCS-induced activation of human PXR and CAR has a greater role in producing downstream up-regulation of hepatic catabolism. Further, these *in vitro* receptor reporter models do not necessarily take into account potential interactions with other receptors and/or coactivators/corepressors that may be important for mediating the total biological response to

TCS; these interactions are presently an unknown variable in this model. Potential involvement of the glucocorticoid receptor (GR) has been ignored in the current approach; the GR is known to regulate the expression and availability of CAR, PXR, and RXR in human hepatocytes (Pascussi *et al.*, 2003; Pascussi *et al.*, 2001; Wang *et al.*, 2003b). Hepatic nuclear factor 4a (HNFa), another orphan nuclear receptor, may be a critical co-regulator of Cyp3a activation via binding to a *cis* promoter element in conjunction with PXR or CAR binding (Jover *et al.*, 2009; Tirona *et al.*, 2003) in human hepatocyte models. This hypothesis introduces the possibility that the importance of co-regulatory transcription factors vary in importance by species. TCS may also affect co-activator recruitment; an interesting example in the literature is guggulsterone, which when co-administered to PXR-null mice with TCPOBOP, a potent mouse CAR agonist, produced decreased expression of Cyp2b10; a current hypothesis for this action if that guggulsterone preferentially recruits the co-activator SRC-1 to PXR and displaces SRC-1 from mouse CAR (Ding *et al.*, 2005; Pascussi *et al.*, 2008). The complexity of nuclear receptor and transcription factor interactions clearly emphasizes that a lack of agonism in the rPXR assay does not eliminate the possibility of rPXR involvement in mediating the *in vivo* response to TCS. Determination of the cumulative result of potential activation of multiple human receptors, e.g. up-regulation of markers of Phase I and Phase II metabolism, necessitates use of a primary hepatocyte model.

6. Conclusions

The human nuclear receptor data presented in the current work demonstrate that TCS is capable of activating hPXR, hCAR2, and hCAR3. These receptors are known to be involved in the transcriptional regulation of Phase I, II, and III xenobiotic metabolizing enzymes and hepatic transporters that help maintain thyroid hormone homeostasis. TCS acts as an inverse

agonist in the rCAR and mCAR reporter assays, suggesting that the *in vivo* effects of TCS may be due to CAR activation in the rat in a manner similar to PB. Based on these nuclear receptor actions, the hazard of TCS-induced hypothyroxinemia, as observed *in vivo* in the rat, seems plausible in a human model. These results indicate that up-regulation of important Phase I markers and Phase II metabolizing enzymes may occur in both humans and rats, but perhaps with species-dependent bias toward the nuclear receptor most involved: both CAR and PXR may be important for mediating downstream responses in humans, and CAR may be more important for the rat.

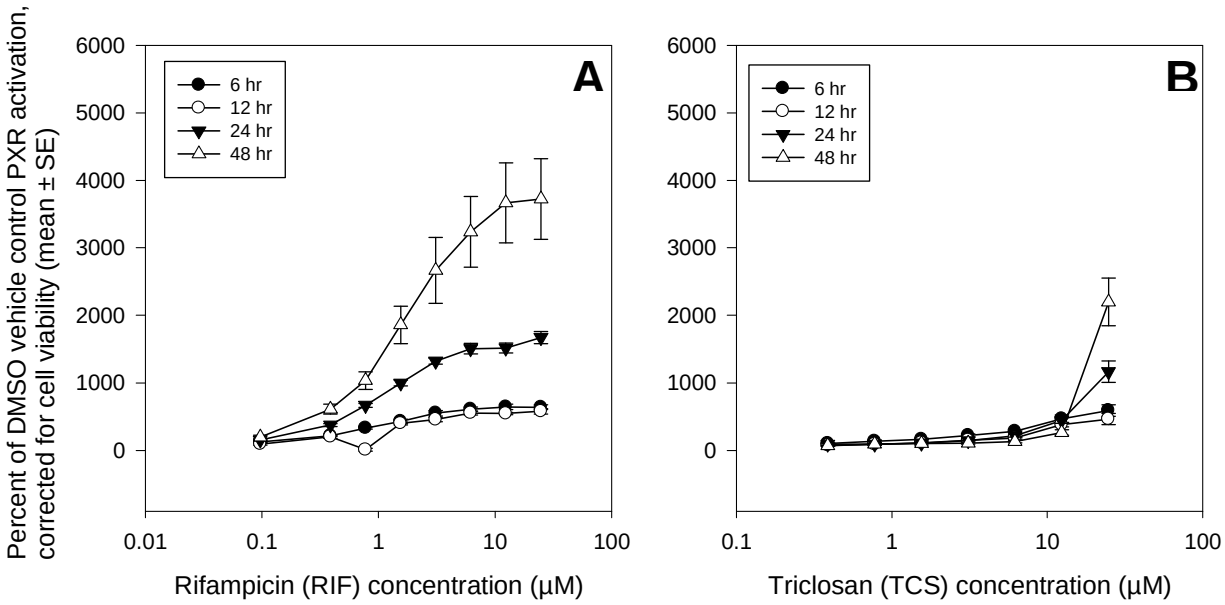


Figure 5.1. hPXR activation in DPX2 cells at 6, 12, 24, and 48 hr of chemical exposure. A. RIF induction over a 48 hr period with concentrations spanning 0.0968 to 24.8 µM. B. TCS induction over a 48 hr period with concentrations spanning 0.387 to 24.8 µM. Using the molecular weight of TCS (289.54 g/mol), the *in vitro* TCS concentration range (0.387 to 24.8 µM) can be expressed as 0.112 to 7.18 mg/L. The *in vivo* doses used in Chapters 2, 3, and 4 of this dissertation work, 10 to 1000 mg/kg/day, can similarly be converted to 34 to 3400 µmol/kg/day.

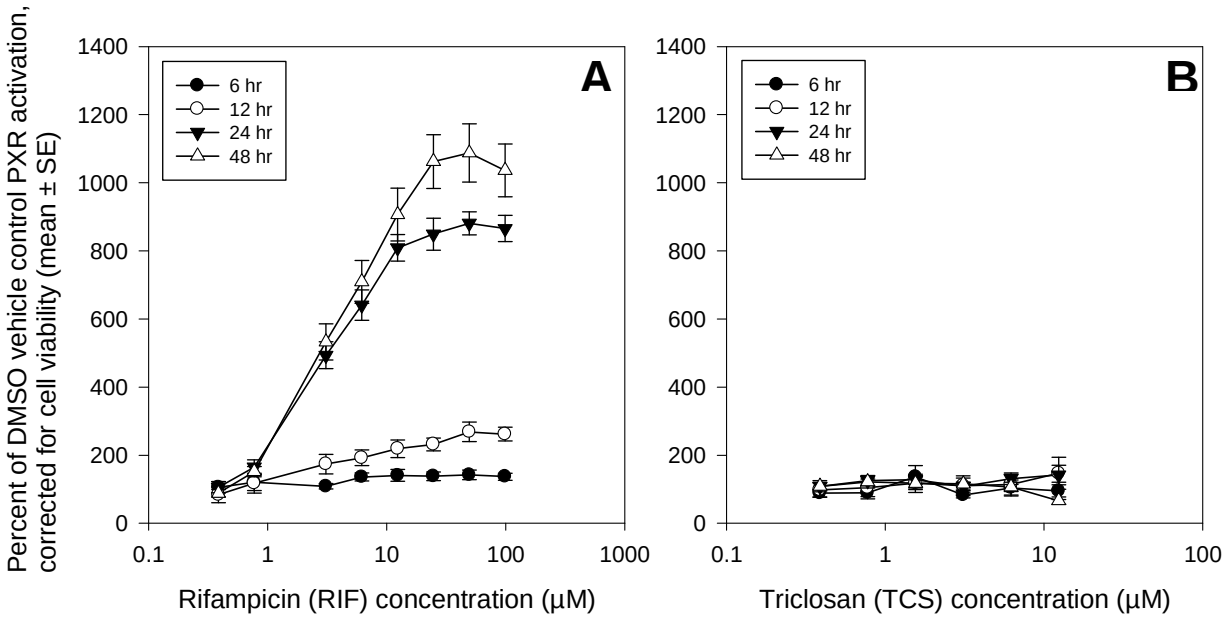


Figure 5.2. rPXR activation in RPXR cells at 6, 12, 24, and 48 hr of chemical exposure. A. DEX induction over a 48 hr period with concentrations spanning 0.387 to 99.1 µM. B. TCS induction over a 48 hr period with concentrations spanning 0.387 to 12.4 µM.

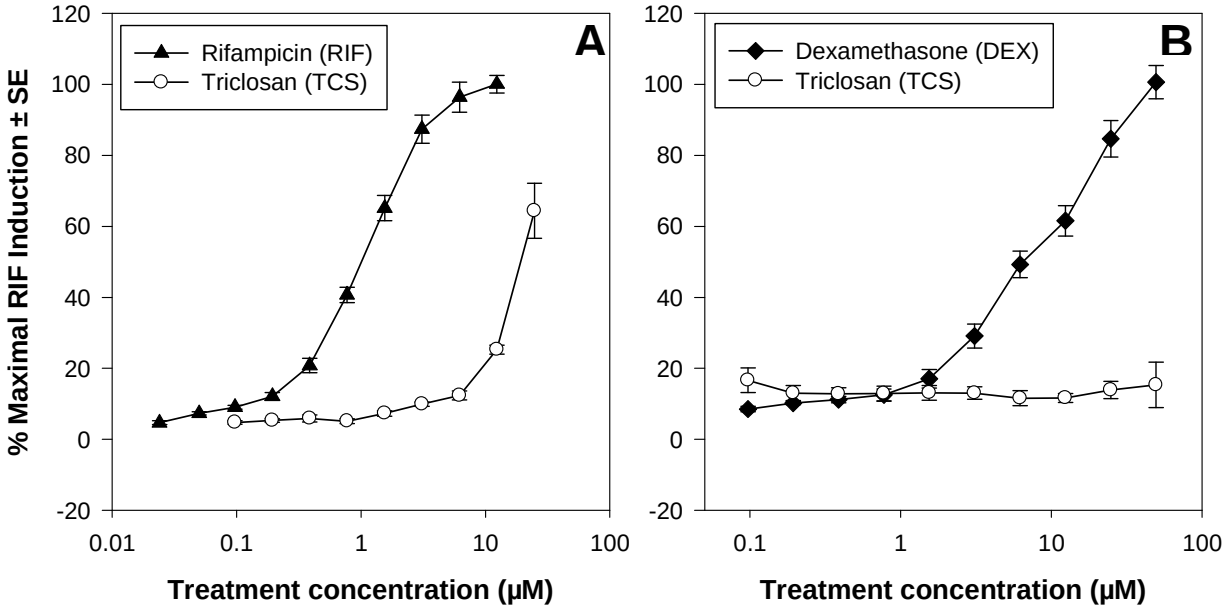


Figure 5.3. Comparative activation of full-length PXR for human and rat. A. RIF and TCS activated hPXR. B. DEX activated rPXR, while TCS failed to activate at any dose.

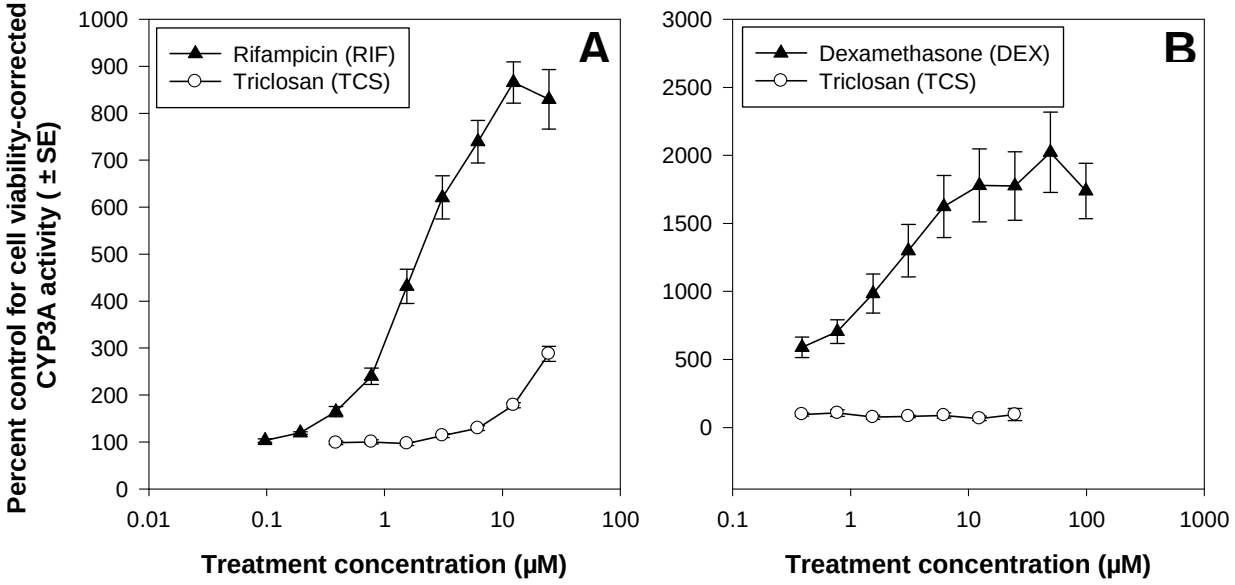


Figure 5.4. Comparative CYP3A4 activation for human and rat in the full-length receptor reporter cell lines. A. RIF and TCS induced CYP3A4 activity in DPX-2 cells. B. DEX induced CYP3A4, and TCS did not produce any effect.

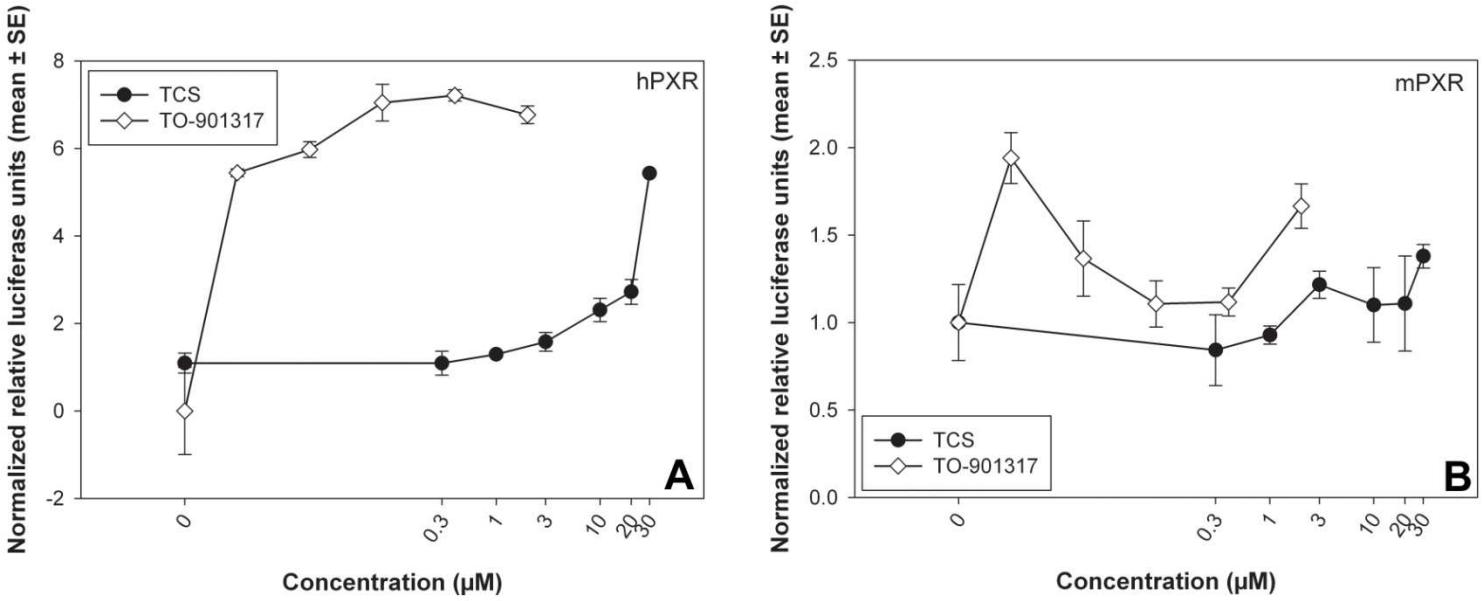


Figure 5.5. Activation of chimeric human and mouse PXR. A. TO-901317 and TCS both activated hPXR. B. TO-901317 activated mPXR significantly at 2 µM, but TCS failed to significantly activate mPXR. Using the molecular weight of TCS (289.54 g/mol), the *in vitro* TCS concentration range (0.3 to 30 µM) can be expressed as 0.087 to 8.7 mg/L. The *in vivo* doses used in Chapters 2, 3, and 4 of this dissertation work, 10 to 1000 mg/kg/day, can similarly be converted to 34 to 3400 µmol/kg/day.

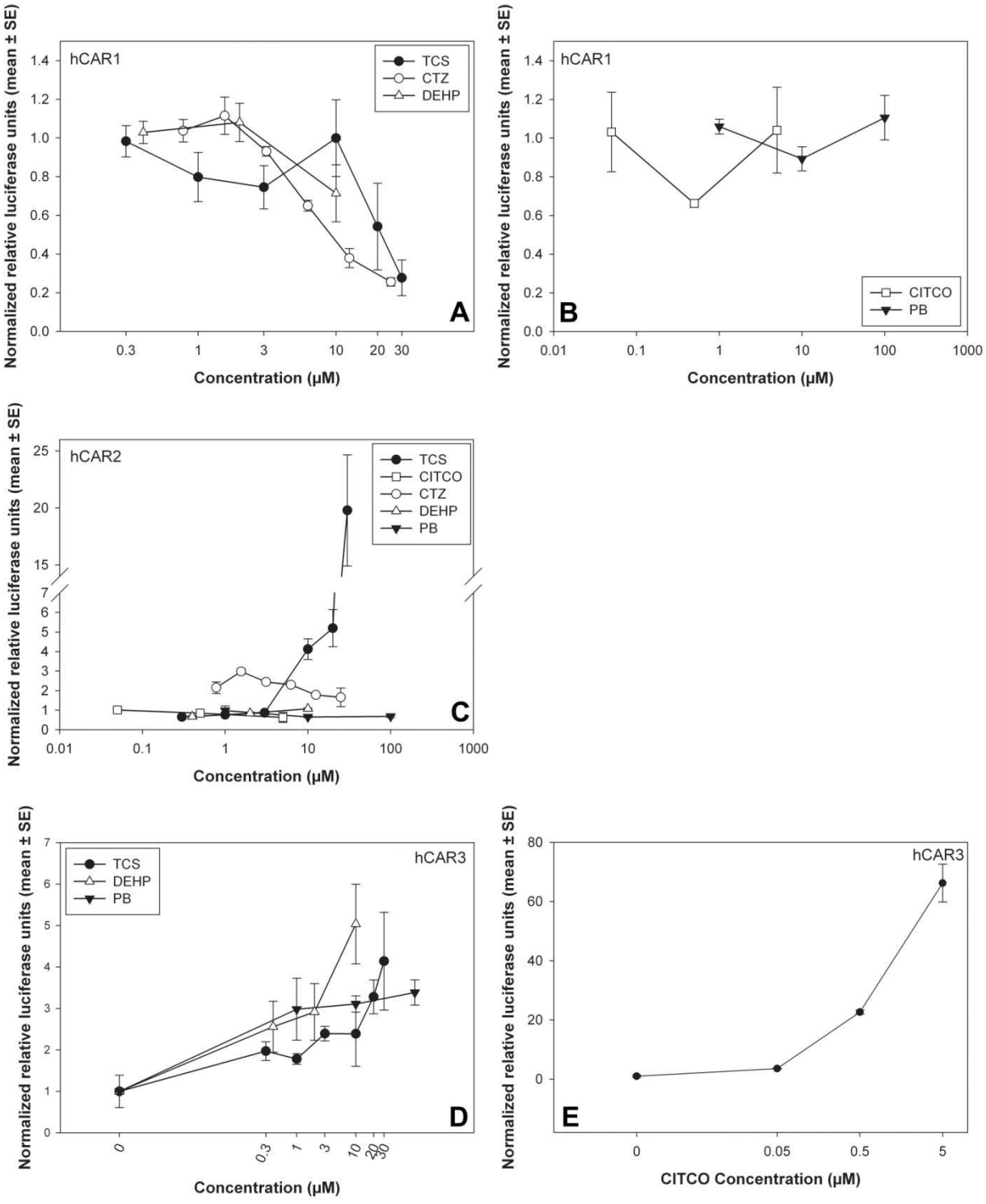

hCAR1
TCS
CTZ
DEHP
A
Normalized relative luciferase units (mean ± SE)
Concentration (μM)
hCAR1
CITCO
PB
B
hCAR2
TCS
CITCO
CTZ
DEHP
PB
C
hCAR3
TCS
DEHP
PB
D
hCAR3
E
CITCO Concentration (μM)

Figure 5.6. Activation of the chimeric hCAR by splice variant. A and B. TCS, CTZ, and DEHP were inverse agonists of hCAR1, while CITCO and PB appeared to have no activity at the receptor. C. TCS demonstrated significant and strong agonism of hCAR2; CTZ demonstrated moderately increased activation of hCAR2 that was not dose-dependent; DEHP, CITCO, and PB failed to activate hCAR2. D and E. TCS, DEHP, and PB were moderate agonists of hCAR3, while CITCO demonstrated potent and efficacious agonism.

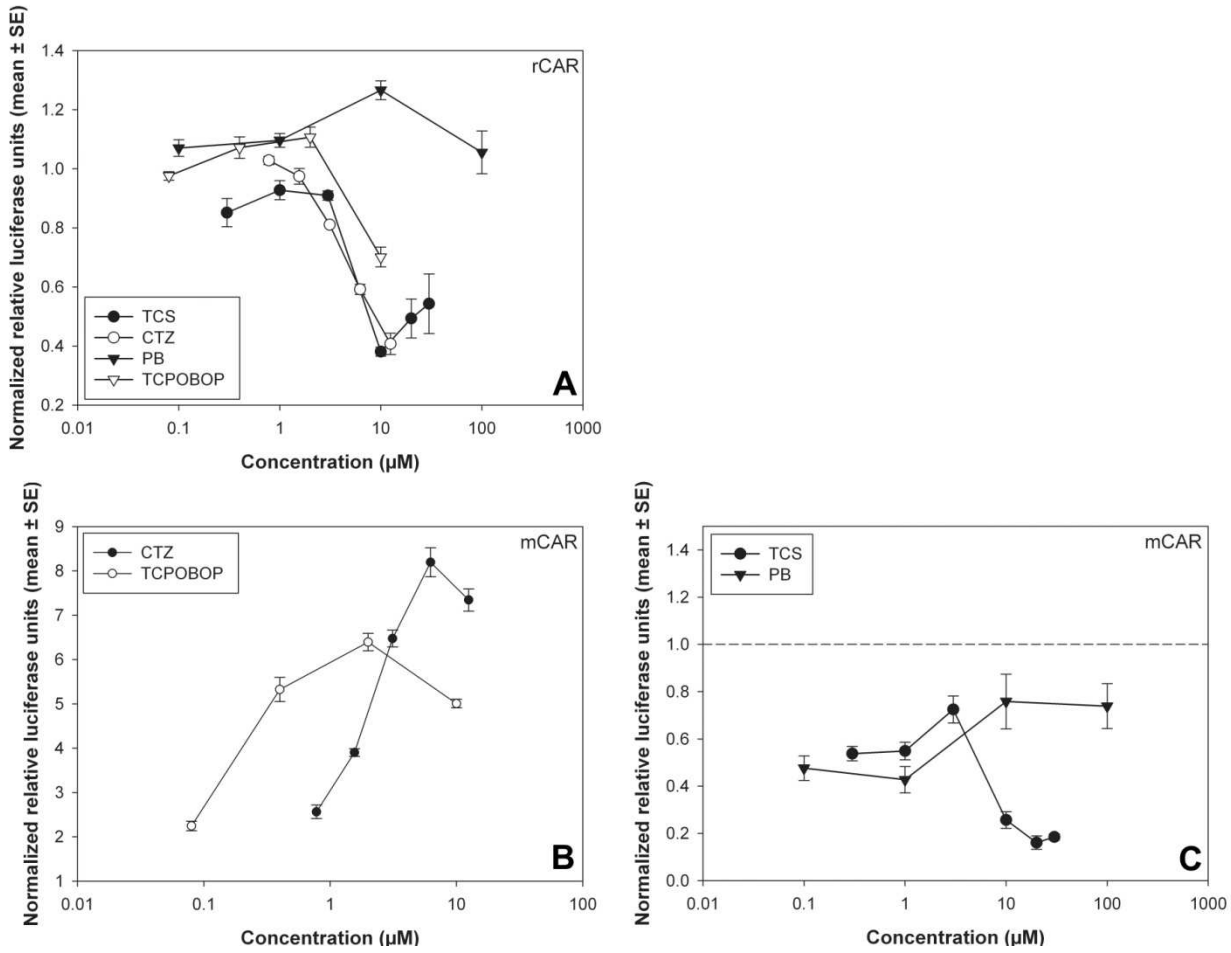


Figure 5.7. Activation of chimeric rat and mouse CAR. A. TCS, CTZ, and TCPOBOP demonstrated inverse agonism toward rCAR, while PB demonstrated no activity. B and C. CTZ and TCPOBOP were agonists of mCAR, whereas TCS and PB demonstrated inverse agonism of mCAR.

References

Auerbach, S. S., R. Ramsden, M. A. Stoner, C. Verlinde, C. Hassett and C. J. Omiecinski (2003). Alternatively spliced isoforms of the human constitutive androstane receptor. *Nucleic Acids Res* **31**, 3194-3207.

Auerbach, S. S., M. A. Stoner, S. Su and C. J. Omiecinski (2005). Retinoid X receptor-alpha-dependent transactivation by a naturally occurring structural variant of human constitutive androstane receptor (NR1I3). *Molecular pharmacology* **68**, 1239-1253.

Auerbach, S. S., J. G. Dekeyser, M. A. Stoner and C. J. Omiecinski (2007). CAR2 displays unique ligand binding and RXRalpha heterodimerization characteristics. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 428-439.

Auso, E., R. Lavado-Autric, E. Cuevas, F. E. Del Rey, G. Morreale De Escobar and P. Berbel (2004). A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration. *Endocrinology* **145**, 4037-4047.

Baldwin, W. S. and J. A. Roling (2008). A Concentration Addition Model for the Activation of the Constitutive Androstane Receptor by Xenobiotic Mixtures. *Toxicol Sci*.

Barter, R. A. and C. D. Klaassen (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. *Toxicology and applied pharmacology* **128**, 9-17.

Boas, M., U. Feldt-Rasmussen, N. E. Skakkebaek and K. M. Main (2006). Environmental chemicals and thyroid function. *European journal of endocrinology / European Federation of Endocrine Societies* **154**, 599-611.

Boobis, A. R., J. E. Doe, B. Heinrich-Hirsch, M. E. Meek, S. Munn, M. Ruchirawat, J. Schlatter, J. Seed and C. Vickers (2008). IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Critical reviews in toxicology* **38**, 87-96.

Buckley, D. B. and C. D. Klaassen (2009). Induction of mouse UDP-glucuronosyltransferase mRNA expression in liver and intestine by activators of aryl-hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, peroxisome proliferator-activated receptor alpha, and nuclear factor erythroid 2-related factor 2. *Drug metabolism and disposition: the biological fate of chemicals* **37**, 847-856.

Burk, O., K. A. Arnold, A. K. Nussler, E. Schaeffeler, E. Efimova, B. A. Avery, M. A. Avery, M. F. Fromm and M. Eichelbaum (2005). Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Molecular pharmacology* **67**, 1954-1965.

Capen, C. C. (1994). Mechanisms of chemical injury of thyroid gland. *Progress in clinical and biological research* **387**, 173-191.

Chang, T. K. and D. J. Waxman (2006). Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev* **38**, 51-73.

Chen, C., J. L. Staudinger and C. D. Klaassen (2003). Nuclear receptor, pregname X receptor, is required for induction of UDP-glucuronosyltranferases in mouse liver by pregnenolone-16 alpha-carbonitrile. *Drug metabolism and disposition: the biological fate of chemicals* **31**, 908-915.

Chen, T., L. M. Tompkins, L. Li, H. Li, G. Kim, Y. Zheng and H. Wang (2010). A single amino acid controls the functional switch of human constitutive androstane receptor (CAR) 1 to the xenobiotic-sensitive splicing variant CAR3. *The Journal of pharmacology and experimental therapeutics* **332**, 106-115.

Cheng, X., J. Maher, M. Z. Dieter and C. D. Klaassen (2005). Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1276-1282.

Cheng, X. and C. D. Klaassen (2006). Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine. *Drug metabolism and disposition: the biological fate of chemicals* **34**, 1863-1867.

Crofton, K. M., K. B. Paul, J. M. Hedge and M. J. DeVito (2007). Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine. *Environ Toxicol Pharmacol* **24**, 194-197.

Crofton, K. M. (2008). Thyroid disrupting chemicals: mechanisms and mixtures. *International journal of andrology* **31**, 209-223.

DeKeyser, J. G., M. C. Stagliano, S. S. Auerbach, K. S. Prabhu, A. D. Jones and C. J. Omiecinski (2009). Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant CAR2. *Molecular pharmacology* **75**, 1005-1013.

Dekeyser, J. G., E. M. Laurenzana, E. C. Peterson, T. Chen and C. J. Omiecinski (2011). Selective phthalate activation of naturally occurring human constitutive androstane receptor splice variants and the pregnane X receptor. *Toxicol Sci*.

DeVito, M., L. Biegel, A. Brouwer, S. Brown, F. Brucker-Davis, A. O. Cheek, R. Christensen, T. Colborn, P. Cooke, J. Crissman, K. Crofton, D. Doerge, E. Gray, P. Hauser, P. Hurley, M. Kohn, J. Lazar, S. McMaster, M. McClain, E. McConnell, C. Meier, R. Miller, J. Tietge and R. Tyl (1999). Screening methods for thyroid hormone disruptors. *Environmental health perspectives* **107**, 407-415.

Ding, X. and J. L. Staudinger (2005). The ratio of constitutive androstane receptor to pregnane X receptor determines the activity of guggulsterone against the Cyp2b10 promoter. *The Journal of pharmacology and experimental therapeutics* **314**, 120-127.

Faucette, S. R., T. Sueyoshi, C. M. Smith, M. Negishi, E. L. Lecluyse and H. Wang (2006). Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *The Journal of pharmacology and experimental therapeutics* **317**, 1200-1209.

Faucette, S. R., T. C. Zhang, R. Moore, T. Sueyoshi, C. J. Omiecinski, E. L. LeCluyse, M. Negishi and H. Wang (2007). Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *The Journal of pharmacology and experimental therapeutics* **320**, 72-80.

Fery, Y., S. O. Mueller and D. Schrenk (2010). Development of stably transfected human and rat hepatoma cell lines for the species-specific assessment of xenobiotic response enhancer module (XREM)-dependent induction of drug metabolism. *Toxicology* **277**, 11-19.

Geick, A., M. Eichelbaum and O. Burk (2001). Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* **276**, 14581-14587.

Gilbert, M. E., W. R. Mundy and K. M. Crofton (2000). Spatial learning and long-term potentiation in the dentate gyrus of the hippocampus in animals developmentally exposed to Aroclor 1254. *Toxicol Sci* **57**, 102-111.

Gilbert, M. E. and L. Sui (2006). Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. *Brain Res* **1069**, 10-22.

Goldey, E. S., L. S. Kehn, G. L. Rehnberg and K. M. Crofton (1995). Effects of developmental hypothyroidism on auditory and motor function in the rat. *Toxicology and applied pharmacology* **135**, 67-76.

Goldey, E. S. and K. M. Crofton (1998). Thyroxine replacement attenuates hypothyroxinemia, hearing loss, and motor deficits following developmental exposure to Aroclor 1254 in rats. *Toxicol Sci* **45**, 94-105.

Goodwin, B., L. B. Moore, C. M. Stoltz, D. D. McKee and S. A. Kliewer (2001). Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Molecular pharmacology* **60**, 427-431.

Guo, G. L., D. R. Johnson and C. D. Klaassen (2002a). Postnatal expression and induction by pregnenolone-16alpha-carbonitrile of the organic anion-transporting polypeptide 2 in rat liver. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 283-288.

Guo, G. L., J. Staudinger, K. Ogura and C. D. Klaassen (2002b). Induction of rat organic anion transporting polypeptide 2 by pregnenolone-16alpha-carbonitrile is via interaction with pregnane X receptor. *Molecular pharmacology* **61**, 832-839.

Guo, G. L., G. Lambert, M. Negishi, J. M. Ward, H. B. Brewer, Jr., S. A. Kliewer, F. J. Gonzalez and C. J. Sinal (2003). Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem* **278**, 45062-45071.

Haddow, J. E., G. E. Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *The New England journal of medicine* **341**, 549-555.

Hanioka, N., H. Jinno, T. Nishimura and M. Ando (1997). Effect of 2,4,4'-trichloro-2'-hydroxydiphenyl ether on cytochrome P450 enzymes in the rat liver. *Chemosphere* **34**, 719-730.

Hood, A., M. L. Allen, Y. Liu, J. Liu and C. D. Klaassen (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicology and applied pharmacology* **188**, 6-13.

Istrate, M. A., A. K. Nussler, M. Eichelbaum and O. Burk (2010). Regulation of CYP3A4 by pregnane X receptor: The role of nuclear receptors competing for response element binding. *Biochemical and biophysical research communications* **393**, 688-693.

Iyer, M., E. J. Reschly and M. D. Krasowski (2006). Functional evolution of the pregnane X receptor. *Expert Opin Drug Metab Toxicol* **2**, 381-397.

Jacobs, M. N., G. T. Nolan and S. R. Hood (2005). Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicology and applied pharmacology* **209**, 123-133.

Jansen, J., E. C. Friesema, C. Milici and T. J. Visser (2005). Thyroid hormone transporters in health and disease. *Thyroid* **15**, 757-768.

Jinno, H., N. Hanioka, S. Onodera, T. Nishimura and M. Ando (1997). Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel. *Xenobiotica; the fate of foreign compounds in biological systems* **27**, 681-692.

Jinno, H., T. Tanaka-Kagawa, N. Hanioka, S. Ishida, M. Saeki, A. Soyama, M. Itoda, T. Nishimura, Y. Saito, S. Ozawa, M. Ando and J. Sawada (2004). Identification of novel alternative splice variants of human constitutive androstane receptor and characterization of their expression in the liver. *Molecular pharmacology* **65**, 496-502.

Jones, S. A., L. B. Moore, J. L. Shenk, G. B. Wisely, G. A. Hamilton, D. D. McKee, N. C. Tomkinson, E. L. LeCluyse, M. H. Lambert, T. M. Willson, S. A. Kliewer and J. T. Moore (2000). The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Molecular endocrinology (Baltimore, Md* **14**, 27-39.

Jover, R., M. Moya and M. J. Gomez-Lechon (2009). Transcriptional regulation of cytochrome p450 genes by the nuclear receptor hepatocyte nuclear factor 4-alpha. *Current drug metabolism* **10**, 508-519.

Konno, Y., M. Negishi and S. Kodama (2008). The roles of nuclear receptors CAR and PXR in hepatic energy metabolism. *Drug Metab Pharmacokinet* **23**, 8-13.

Kretschmer, X. C. and W. S. Baldwin (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chemico-biological interactions* **155**, 111-128.

Lavado-Autric, R., E. Auso, J. V. Garcia-Velasco, C. Arufe Mdel, F. Escobar del Rey, P. Berbel and G. Morreale de Escobar (2003). Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. *The Journal of clinical investigation* **111**, 1073-1082.

LeCluyse, E. L. (2001). Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chemico-biological interactions* **134**, 283-289.

Lehmann, J. M., D. D. McKee, M. A. Watson, T. M. Willson, J. T. Moore and S. A. Kliewer (1998). The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *The Journal of clinical investigation* **102**, 1016-1023.

Liu, J., Y. Liu, R. A. Barter and C. D. Klaassen (1995). Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *The Journal of pharmacology and experimental therapeutics* **273**, 977-985.

Luo, G., M. Cunningham, S. Kim, T. Burn, J. Lin, M. Sinz, G. Hamilton, C. Rizzo, S. Jolley, D. Gilbert, A. Downey, D. Mudra, R. Graham, K. Carroll, J. Xie, A. Madan, A. Parkinson, D. Christ, B. Selling, E. LeCluyse and L. S. Gan (2002). CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 795-804.

Maglich, J. M., C. M. Stoltz, B. Goodwin, D. Hawkins-Brown, J. T. Moore and S. A. Kliewer (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Molecular pharmacology* **62**, 638-646.

Maglich, J. M., D. J. Parks, L. B. Moore, J. L. Collins, B. Goodwin, A. N. Billin, C. A. Stoltz, S. A. Kliewer, M. H. Lambert, T. M. Willson and J. T. Moore (2003). Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* **278**, 17277-17283.

Maglich, J. M., J. Watson, P. J. McMillen, B. Goodwin, T. M. Willson and J. T. Moore (2004). The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. *J Biol Chem* **279**, 19832-19838.

Maglich, J. M., D. C. Lobe and J. T. Moore (2009). The nuclear receptor CAR (NR1I3) regulates serum triglyceride levels under conditions of metabolic stress. *J Lipid Res* **50**, 439-445.

Miller, M. D., K. M. Crofton, D. C. Rice and R. T. Zoeller (2009). Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. *Environmental health perspectives* **117**, 1033-1041.

Mitro, N., L. Vargas, R. Romeo, A. Koder and E. Saez (2007). T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS Lett* **581**, 1721-1726.

Moore, L. B., D. J. Parks, S. A. Jones, R. K. Bledsoe, T. G. Consler, J. B. Stimmel, B. Goodwin, C. Liddle, S. G. Blanchard, T. M. Willson, J. L. Collins and S. A. Kliewer (2000). Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**, 15122-15127.

Moore, L. B., J. M. Maglich, D. D. McKee, B. Wisely, T. M. Willson, S. A. Kliewer, M. H. Lambert and J. T. Moore (2002). Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Molecular endocrinology (Baltimore, Md* **16**, 977-986.

Mutoh, S., M. Osabe, K. Inoue, R. Moore, L. Pedersen, L. Perera, Y. Rebolloso, T. Sueyoshi and M. Negishi (2009). Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). *J Biol Chem* **284**, 34785-34792.

Opazo, M. C., A. Gianini, F. Pancetti, G. Azkcona, L. Alarcon, R. Lizana, V. Noches, P. A. Gonzalez, M. P. Marassi, S. Mora, D. Rosenthal, E. Eugenin, D. Naranjo, S. M. Bueno, A. M. Kalergis and C. A. Riedel (2008). Maternal hypothyroxinemia impairs spatial learning and synaptic nature and function in the offspring. *Endocrinology* **149**, 5097-5106.

Pascussi, J. M., L. Drocourt, S. Gerbal-Chaloin, J. M. Fabre, P. Maurel and M. J. Vilarem (2001). Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. *Eur J Biochem* **268**, 6346-6358.

Pascussi, J. M., M. Busson-Le Coniat, P. Maurel and M. J. Vilarem (2003). Transcriptional analysis of the orphan nuclear receptor constitutive androstane receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. *Molecular endocrinology (Baltimore, Md* **17**, 42-55.

Pascussi, J. M., S. Gerbal-Chaloin, C. Duret, M. Daujat-Chavanieu, M. J. Vilarem and P. Maurel (2008). The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. *Annu Rev Pharmacol Toxicol* **48**, 1-32.

Paul, K. B., J. M. Hedge, M. J. Devito and K. M. Crofton (2010a). Developmental triclosan exposure decreases maternal and neonatal thyroxine in rats. *Environmental toxicology and chemistry / SETAC* **29**, 2840-2844.

Paul, K. B., J. M. Hedge, M. J. DeVito and K. M. Crofton (2010b). Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol Sci* **113**, 367-379.

Pop, V. J., J. L. Kuijpens, A. L. van Baar, G. Verkerk, M. M. van Son, J. J. de Vijlder, T. Vulsma, W. M. Wiersinga, H. A. Drexhage and H. L. Vader (1999). Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clinical endocrinology* **50**, 149-155.

Pop, V. J., E. P. Brouwers, H. L. Vader, T. Vulsma, A. L. van Baar and J. J. de Vijlder (2003). Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clinical endocrinology* **59**, 282-288.

Qatanani, M. and D. D. Moore (2005a). CAR, the continuously advancing receptor, in drug metabolism and disease. *Current drug metabolism* **6**, 329-339.

Qatanani, M., J. Zhang and D. D. Moore (2005b). Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* **146**, 995-1002.

Ren, H., L. M. Aleksunes, C. Wood, B. Vallanat, M. H. George, C. D. Klaassen and J. C. Corton (2010). Characterization of peroxisome proliferator-activated receptor alpha--independent effects of PPARalpha activators in the rodent liver: di-(2-ethylhexyl) phthalate also activates the constitutive-activated receptor. *Toxicol Sci* **113**, 45-59.

Ross, J., S. M. Plummer, A. Rode, N. Scheer, C. C. Bower, O. Vogel, C. J. Henderson, C. R. Wolf and C. R. Elcombe (2010). Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogens phenobarbital and chlordane in vivo. *Toxicol Sci* **116**, 452-466.

Savkur, R. S., Y. Wu, K. S. Bramlett, M. Wang, S. Yao, D. Perkins, M. Totten, G. Searfoss, 3rd, T. P. Ryan, E. W. Su and T. P. Burris (2003). Alternative splicing within the ligand binding domain of the human constitutive androstane receptor. *Mol Genet Metab* **80**, 216-226.

Schuur, A. G., F. M. Boekhorst, A. Brouwer and T. J. Visser (1997). Extrathyroidal effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on thyroid hormone turnover in male Sprague-Dawley rats. *Endocrinology* **138**, 3727-3734.

Sinz, M. W., D. Pray and J. Raucy (2007). The utility of stable cell lines to assess species differences in PXR transactivation. *Drug Metab Lett* **1**, 147-152.

Smirlis, D., R. Muangmoonchai, M. Edwards, I. R. Phillips and E. A. Shephard (2001). Orphan receptor promiscuity in the induction of cytochromes p450 by xenobiotics. *J Biol Chem* **276**, 12822-12826.

Staudinger, J. L., B. Goodwin, S. A. Jones, D. Hawkins-Brown, K. I. MacKenzie, A. LaTour, Y. Liu, C. D. Klaassen, K. K. Brown, J. Reinhard, T. M. Willson, B. H. Koller and S. A. Kliewer (2001). The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98**, 3369-3374.

Sueyoshi, T., T. Kawamoto, I. Zelko, P. Honkakoski and M. Negishi (1999). The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274**, 6043-6046.

Sueyoshi, T. and M. Negishi (2001). Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* **41**, 123-143.

Tien, E. S., D. B. Hannon, J. T. Thompson and J. P. Vanden Heuvel (2006). Examination of Ligand-Dependent Coactivator Recruitment by Peroxisome Proliferator-Activated Receptor-alpha (PPARalpha). *PPAR Res* **2006**, 69612.

Timsit, Y. E. and M. Negishi (2007). CAR and PXR: the xenobiotic-sensing receptors. *Steroids* **72**, 231-246.

Tirona, R. G., W. Lee, B. F. Leake, L. B. Lan, C. B. Cline, V. Lamba, F. Parviz, S. A. Duncan, Y. Inoue, F. J. Gonzalez, E. G. Schuetz and R. B. Kim (2003). The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**, 220-224.

Tzameli, I., P. Pissios, E. G. Schuetz and D. D. Moore (2000). The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* **20**, 2951-2958.

Urquhart, B. L., R. G. Tirona and R. B. Kim (2007). Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol* **47**, 566-578.

Vanden Heuvel, J. P., J. T. Thompson, S. R. Frame and P. J. Gillies (2006). Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-alpha, -beta, and -gamma, liver X receptor-beta, and retinoid X receptor-alpha. *Toxicol Sci* **92**, 476-489.

Vansell, N. R. and C. D. Klaassen (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicology and applied pharmacology* **176**, 187-194.

Vansell, N. R. and C. D. Klaassen (2002). Increase in rat liver UDP-glucuronosyltransferase mRNA by microsomal enzyme inducers that enhance thyroid hormone glucuronidation. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 240-246.

Vermiglio, F., V. P. Lo Presti, M. Moleti, M. Sidoti, G. Tortorella, G. Scaffidi, M. G. Castagna, F. Mattina, M. A. Violi, A. Crisa, A. Artemisia and F. Trimarchi (2004). Attention deficit and hyperactivity disorders in the offspring of mothers exposed to mild-moderate iodine deficiency: a possible novel iodine deficiency disorder in developed countries. *J Clin Endocrinol Metab* **89**, 6054-6060.

Wang, H., S. Faucette, T. Sueyoshi, R. Moore, S. Ferguson, M. Negishi and E. L. LeCluyse (2003a). A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* **278**, 14146-14152.

Wang, H., S. R. Faucette, D. Gilbert, S. L. Jolley, T. Sueyoshi, M. Negishi and E. L. LeCluyse (2003b). Glucocorticoid receptor enhancement of pregnane X receptor-mediated CYP2B6 regulation in primary human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **31**, 620-630.

Wang, H. and E. L. LeCluyse (2003c). Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin Pharmacokinet* **42**, 1331-1357.

Wang, H., S. Faucette, R. Moore, T. Sueyoshi, M. Negishi and E. LeCluyse (2004). Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. *J Biol Chem* **279**, 29295-29301.

Wang, L. Q. and M. O. James (2006). Inhibition of sulfotransferases by xenobiotics. *Current drug metabolism* **7**, 83-104.

Watkins, R. E., J. M. Maglich, L. B. Moore, G. B. Wisely, S. M. Noble, P. R. Davis-Searles, M. H. Lambert, S. A. Kliewer and M. R. Redinbo (2003). 2.1 A crystal structure of human PXR in complex with the St. John's wort compound hyperforin. *Biochemistry* **42**, 1430-1438.

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

Yen, P. M. (2001). Physiological and molecular basis of thyroid hormone action. *Physiol Rev* **81**, 1097-1142.

Yueh, M. F., M. Kawahara and J. Raucy (2005). High volume bioassays to assess CYP3A4-mediated drug interactions: induction and inhibition in a single cell line. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 38-48.

Zhang, J., W. Huang, M. Qatanani, R. M. Evans and D. D. Moore (2004). The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. *J Biol Chem* **279**, 49517-49522.

Zhou, J., J. Zhang and W. Xie (2005). Xenobiotic nuclear receptor-mediated regulation of UDP-glucuronosyl-transferases. *Current drug metabolism* **6**, 289-298.

Zoeller, R. T., S. W. Tan and R. W. Tyl (2007). General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Critical reviews in toxicology* **37**, 11-53.

Zorrilla, L. M., E. K. Gibson, S. C. Jeffay, K. M. Crofton, W. R. Setzer, R. L. Cooper and T. E. Stoker (2009). The effects of triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol Sci* **107**, 56-64.

Summary

Chapter 6

Katie B. Paul[1]

[1]University of North Carolina at Chapel Hill, Curriculum in Toxicology, CB 7270, Chapel Hill, NC 27599

The current work characterized a mode-of-action (MOA) for triclosan- (TCS) induced decreases in thyroid hormones (THs) in rats, and investigated the human relevance of this MOA. TCS is an active anti-bacterial agent found in a variety of personal care products and medical topical disinfectants. In the last decade, analytical chemistry advances identified a set of emerging contaminants of concern, which includes TCS (reference on emerging contaminants). Detection of TCS by a USGS team in surface waters across the United States demonstrated that TCS was one of the most ubiquitously detected contaminants, found at over 50% of the sites above the limit of detection, though at low concentrations in the parts per trillion range on average (50 ng/L) (Kolpin *et al.*, 2002). Though systemically toxic to algae, macroinvertebrates, and fish, an understanding of how the combined environmental and hygienic exposure to TCS could impact the human thyroid hormone (TH) homeostasis had not been evaluated. Limited evidence from the literature suggested that TCS might be a candidate thyroid hormone disruptor: TCS increased the rate of triiodothyronine- (T3) mediated anuran metamorphosis (Veldhoen *et al.*, 2006), moderately activated the human pregnane X receptor (PXR) in an *in vitro* receptor reporter assay (Jacobs *et al.*, 2005), increased protein expression and activity in primary rat hepatocytes (Jinno *et al.*, 1997) as well as rat hepatic microsomes prepared following *in vivo* exposures (Hanioka *et al.*, 1997), and inhibited sulfotransferases *in vitro* (Wang *et al.*, 2004; Wang *et al.*, 2006). A clear data gap and subsequent hypothesis was identified based on these published results: TCS alters TH regulation, and in mammals does so via interaction with nuclear receptors, resulting in increased hepatic Phase II metabolism and transport. This research tested the hypothesis that TCS decreases thyroid hormones *in vivo* via activation of nuclear receptors that regulate key hepatic enzymatic regulators of TH homeostasis. Extension of this work to a developmental exposure model addressed the capacity for TH disruption by TCS during neurodevelopment.

Finally, using the hypothesized MOA, the initiating key event and biomarkers of hepatic catabolism were assessed for plausibility using comparative *in vitro* models for rats and humans.

Chapter 2 of this work demonstrated that short-term TCS exposure decreased thyroxine (T4) and T3 in a dose-dependent manner. The no observable adverse effect level (NOAEL) for T4 was 30 mg/kg/day, and the greatest effect observed as an approximate 55% decrease in T4 with 1000 mg/kg/day TCS. Effects on T3 were minimal, with 12% and 25% decreases at 100 and 300 mg/kg/day, respectively; most likely these were only significant changes due to the large N used in this study. The magnitude of these changes versus the dose administered indicates that TCS is a low potency thyroid hormone disruptor when compared to other xenobiotics. Similar 4-day exposures with other compounds have yielded greater depletion of thyroid hormones. Commercial mixtures of polybrominated diphenyl ethers (PBDEs) DE-71 and DE-79 decreased T4 by 80 and 70% respectively, and T3 by 25-30%, with no effects on TSH, following 4-day exposures of 300 mg/kg/day (Zhou *et al.*, 2001). The NOAEL for these PBDE-mediated effects was 3 mg/kg/day, as T4 was decreased 20-30% even with 10 mg/kg/day (Zhou et al., 2001), indicating that PBDEs disrupt TH homeostasis with higher potency than TCS. Similarly, polychlorinated biphenyls (PCBs) are more potent and more efficacious than TCS; dioxin-like PCBs, e.g. PCB126 which interacts with the aryl hydrocarbon receptor (AhR) to mediate downstream extrathyroidal effects, decreased T4 by 50% in a rat at 30-100 µg/kg/day, and phenobarbital-like PCBs, e.g. PCB153 thought to interact with the constitutive androstane receptor (CAR), decreased T4 by 70-80% with 90-300 mg/kg/day in a rat when exposed for four consecutive days. Thus the consistent finding that TCS decreased T4 by approximately 30% at doses as high as 300 mg/kg/day demonstrates that TCS appears to be relatively less efficacious and less potent

than many environmental thyroid disruptors such as PBDEs and PCBs. Minor decreases in T3 and a lack of consistent, dose-dependent effects on TSH are consistent with other thyroid disrupting chemicals, including 3MC, Aroclor 1254, and phenobarbital (PB) (Hood *et al.*, 1999; Liu *et al.*, 1995; Vansell *et al.*, 2001). One area of uncertainty in the study of thyroid disruption is the correlation, or lack of correlation, between T4 disruption, T3 disruption, and TSH increases; for instance, though Aroclor 1254 is capable of potent disruption of T4, it does not increase TSH (Hood et al., 1999; Liu et al., 1995; Vansell *et al.*, 2001). A predominant theory is that activation of negative feedback to produce TSH increases in response to T4 decreases requires concomitant increases in T3 glucuronidation, and that variability among hepatic microsomal enzyme inducers in their ability to increase glucuronidation of T4 as well as T3 underlies the variability in TSH response (Hood *et al.*, 2000; Richardson *et al.*, 2010a).

Similar to many other environmental chemicals and drugs (Brucker-Davis, 1998), the current body of *in vivo* work presented in Chapters 2 and 4 suggests that TCS exposure decreases T4 *in vivo* via up-regulation of hepatic catabolism. This hypothesized MOA begins with activation of nuclear receptors, including the constitutive androstane receptor and/or pregnane-X receptor (CAR and/or PXR), and followed by a transcriptional up-regulation of hepatic microsomal enzymes responsible for decreasing the serum half-life of T4 (e.g. glucuronyltransferases, sulfotransferases, and hepatic transport proteins). Chapter 2 provides data to suggest that it is plausible that this MOA at least partially accounts for the systemic T4 decreases observed in rats following a 4-day TCS exposure. Both hepatic microsomal activity and quantitative RT-PCR demonstrated isoform-specific increases in Phase I metabolic markers of upstream nuclear receptor activation; pentoxy-o-resorufin deethylase activity, a marker for Cyp2b activity in the rat, and *Cyp2b1/2* and *Cyp3a1/23*

expression, were increased, suggesting that CAR and/or PXR were activated by TCS exposure. Hepatic microsomal T4-glucuronidation activity was increased nearly two-fold at 1000 mg/kg/day only, and qRT-PCR demonstrated two-fold increased expression of *Ugt1a1* at 100-300 mg/kg/day and nearly three-fold increased expression of *Sult1c1*, suggesting that sulfotransferase activity may also have contributed to increased catabolism of thyroid hormones *in vivo*. The potential contribution of nuclear receptor-mediated changes in hepatic transport expression was also evaluated, but qRT-PCR revealed no significant changes despite known regulation of key transporters by CAR and PXR. Follow-up studies of *in vitro* functional activity of hepatic transport in sandwich-cultured rat hepatocytes, presented in Appendix 6, confirmed a lack of significant effect on hepatic transporter protein function and expression following TCS exposure. Collectively, the data suggest that increased T4 glucuronidation and subsequent excretion following short-term TCS exposure, initiated by activation of hepatic nuclear receptors, is plausible in the rat. This follows the MOA pattern for several known and well-characterized thyroid disrupting chemicals, such as PB, PCN, Aroclor 1254 and single PCB congeners, 3MC, and PBDEs. In particular it is important to examine these prototypical inducers with respect to their known receptor targets. In the rat, PB is known to activate CAR; PCN activates PXR; Aroclor 1254 is a mixed inducer containing PCB congeners that activate AhR and/or CAR; 3MC is an AhR agonist; and PBDEs activate CAR and/or PXR. The data presented in Chapter 2 does not delineate the specific nuclear receptor interaction because considerable cross-talk between CAR and PXR could enable either receptor to increase the expression of Cyp2b and Cyp3a activity and expression. However, TCS appears to act upon thyroid hormones in a manner more consistent with CAR activator PB, i.e. moderate effects on T4, minimal effects on T3, and no significant effects on TSH with increases in Cyp2b/Cyp3a markers and T4 glucuronidation

activity following short-term administration. Though the data and comparison to known thyroid-disrupting chemicals illustrates the plausibility of this MOA following short-term exposure in a rat model, a necessary caveat is that T4 glucuronidation may not completely account for the T4 decreases observed. In fact, previous reports of the correlation between T4 glucuronidation activity and T4 decreases for PB and PCN indicated that other events may contribute to the observed TH deceases (Kato *et al.*, 2005; Liu et al., 1995; Richardson *et al.*, 2010b).

Evaluation of this plausible MOA, derived from a short-term exposure model, in a developmental model was a critical component of this work because subclinical maternal hypothyroxinemia results in critically delay neurological and behavioral functions and manifests permanently as decreased cognitive function in both rat (Axelstad *et al.*, 2008; Goldey *et al.*, 1998; Lavado-Autric *et al.*, 2003; Opazo *et al.*, 2008), and human offspring (Berbel *et al.*, 2009; Haddow *et al.*, 1999; Henrichs *et al.*, 2010; Kooistra *et al.*, 2006; Li *et al.*, 2010; Pop *et al.*, 2003; Pop *et al.*, 1999). Thus the developing fetus and neonate represent a susceptible population of concern for TH disruption. For the work detailed in Chapters 3 and 4, perinatal exposure of dams from early gestation through the end of lactation modeled the potential transplacental and lactational exposures to offspring; these routes of exposure likely occur in humans, as TCS has been measured in human plasma (Adolfsson-Erici *et al.*, 2002; Allmyr *et al.*, 2008; Allmyr *et al.*, 2006b) and breast milk (Adolfsson-Erici et al., 2002; Allmyr *et al.*, 2006a; Dayan, 2007). This work tested the hypothesis that TCS decreases thyroid hormones, and that this disruption proceeds through increased hepatic catabolism of thyroid hormones, as with the short-term exposure model. The results presented in Chapters 3 and 4 reveal three key findings: (1) TCS moderately decreased T4 in dams, fetuses, and early neonates, with no effects on thyroid hormones in

pups during mid- to late-lactation (Paul *et al.*, 2010b); (2) Transplacental exposure occurs, and lactation exposure alone produced more limited internal exposures to neonates as they approached weaning; and, (3) markers of up-regulated hepatic catabolism were increased in dams, suggesting that TCS-induced hypothyroxinemia during the fetal and early neonatal period was at least partially due to the previously hypothesized MOA.

The observation that T4 was moderately decreased *in vivo* for dams at gestational day 20 (GD20) and postnatal day 22 (PND22), GD20 fetuses, and PND4 neonates was consistent with the decreases observed in T4 following a 4-day exposure (Paul *et al.*, 2010a). There were no perinatal effects on T3 or TSH for dams or PND14 and PND21 offspring. Serum T4 for dams, fetuses, and PND4 neonates was decreased by approximately 30% for the 300 mg/kg/day exposure group; this finding is particularly interesting because on GD20, dams had received TCS for 14 days, and on PND22, dams had received TCS for 36 days, yet the magnitude of the effect at 300 mg/kg/day was concordant with the effect for 300 mg/kg/day following a 4-day exposure. This could be due to the short half-life for TCS, estimated to be 9-18 hr for oral administration in the rat (personal communication, James Plautz, Ciba Specialty Chemicals) (Black *et al.*, 1975; Siddiqui *et al.*, 1979). This is similar to the 10-24 hr oral half-life reported in humans (Rodricks *et al.*, 2010; Sandborgh-Englund *et al.*, 2006). Thus, following a 4-day exposure, animals may have achieved steady-state kinetics for TCS, such that a 15-day or 36-day exposure would reflect the same internal concentrations of TCS.

The data from Chapters 3 and 4 demonstrated a unique pattern of perinatal TCS exposure on the serum T4 concentrations of pups during the lactation period, i.e. the serum T4 concentrations of pups recovered to control levels by the end of weaning. This pattern suggests that toxicokinetic factors may have affected maternal disposition of triclosan into milk and thereby limited lactation exposure to pups, or that the triclosan may not have

triggered the same toxicodynamic effects in offspring during the lactation period as in exposed weanlings or dams. The work in Chapter 4 tested the hypothesis that the recovery of serum T4 to control levels at PND14 and PND21 in offspring was due to decreased exposure of these pups. As the fetuses and offspring only received TCS via maternal compartments, the similar magnitude of decrease in T4 warranted follow-up analysis of internal TCS exposures to these animals. A lack of TCS-induced hypothyroxinemia on PND14 and PND21 in the offspring makes the effects of perinatal TCS exposure distinct from other known developmental TH disruptors, including polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), propylthiouracil (PTU), and 2,3,7,8-tetrachlorodibenzodioxin (TCDD), which when administered to dams are known to decrease offspring T4 during the entire postnatal lactation period, typically with the most pronounced effects on offspring T4 on PND14 (Crofton *et al.*, 2000; Gilbert *et al.*, 2006; Goldey et al., 1995; Kodavanti *et al.*, 2010; Morse *et al.*, 1993; Royland et al., 2008; Sharlin *et al.*, 2006; Zhou et al., 2001; Zhou et al., 2002). Markers of hepatic Phase I and Phase II catabolic activity are known to be inducible at PND4, 14, and 21 (Zhou et al., 2001; Zhou et al., 2002), suggesting that a differential toxicodynamic response by postnatal age was likely not responsible for the variable T4 response. Based on the lack of effect during these later postnatal offspring ages, a toxicokinetic hypothesis was proposed: PND14 and PND21 neonates recover to vehicle control concentrations of serum T4 because their exposure via lactation is limited.

To evaluate the hypothesis that PND14 and PND21 serum T4 concentrations were unaffected due to limited lactation exposure, parent TCS and parent plus glucuronidated and sulfated TCS were measured in sera and liver homogenates from all of six of the developmental time points by high performance liquid chromatography coupled with tandem

mass spectrometry. The results illustrate that dams experienced the highest internal total TCS concentrations in both sera and liver, with the concentrations slightly higher for GD20 dams, followed closely by GD20 fetuses and then PND4 neonates. A pronounced decrease in total TCS was observed for PND14 and even more so for PND21, indicating that exposure to neonates was decreasing over the lactation period. This supported our hypothesis that the recovery of T4 to control concentrations in PND14 and PND21 neonates corresponded to decreased exposure through lactation. Further, these data suggest that GD20 fetuses and PND4 neonates are not disproportionately susceptible to TCS-induced hypothyroxinemia; similar internal concentrations of TCS produced equivalent T4 reductions in dams, fetuses, and early offspring, such that fetuses and offspring do not appear to be more sensitive to exposure. A potential follow-up to this work would be to test TCS concentrations in the milk of dams, to understand the oral dose being administered to pups, since this was not characterized. TCS has previously been measured in human breast milk (Adolfsson-Erici *et al.*, 2002; Allmyr *et al.*, 2006a; Dayan, 2007). Following oral ingestion of TCS, a predominant exposure route (Sandborgh-Englund et al., 2006), the concentration of TCS in plasma was greater than that of breast milk by a factor of four, possibly due to the relative chemical acidity of the matrices versus the protonation state of TCS (TCS has a pKa of 7.9, serum pH = 7.5, and breast milk pH = 6.5) (Allmyr et al., 2006a). The contribution of other factors that would affect the diffusion of TCS across the mammary membrane, including active transport of TCS into the milk or binding of TCS to serum proteins, is unknown for humans and for rats. Potential interspecies differences in transport of TCS or TCS conjugates into the milk compartment are still an uncertainty in the extrapolation of the rat data to potential human risk; e.g., if rats partition TCS into milk at a greater rate than humans, the risk of human neonatal exposure is further diminished.

Following investigation of the pattern of serum T4 effects versus internal exposures, the hypothesized MOA for TCS-induced hypothyroxinemia was evaluated for plausibility following development exposure. Analysis of the MOA in the developmental model importantly informs whether or not TCS-induced hypothyroxinemia could proceed through this MOA during the critical brain development window, and gives a series of events to evaluate in later in a human model for plausibility. Hepatic microsomal activity assays were used to test up-regulation of markers of Phase I and Phase II activity. Quantitative PCR arrays were employed to evaluate Phase I expression as a marker of upstream nuclear receptor activation, expression of Phase II glucuronsyltransferase and sulfotransferase isoforms, expression of Phase III hepatic transporters, expression of deiodinase I, and markers of tissue changes in T3 (malic enzyme I and Spot14). TCS exposure increased PROD activity for PND22 dams and PND4 neonates only, and T4 glucuronidation activity for PND22 dams, at 300 mg/kg/day. The qPCR arrays demonstrated that expression of *Cyp2b2* and *Cyp3a1/23* was increased by TCS exposure in both sets of dams. However, there were no statistically significant effects for any other expression endpoints. The limited findings are still consistent with activation of CAR and/or PXR, and potential up-regulation of hepatic catabolism, indicating that it is plausible that TCS activates nuclear receptors to result in increased hepatic catabolism of thyroid hormones with subsequent decreases in serum T4 concentrations. The data further confirm that the doses of TCS used in this study, while high (maximum of 300 mg/kg/day), do not significantly perturb tissue concentrations of T3 that would be necessary to elicit more deleterious results during animal development, likely due to the low potency and efficacy of TCS.

Although TCS appears to decrease serum T4 with low potency and efficacy, and perhaps without changing tissue levels of T3, the determination of human relevance of the

proposed MOA is still critical because (1) TCS is only one of the potential thyroid disrupting chemicals that individuals are exposed to; (2) the relative sensitivity of humans and rats to this specific MOA is unknown; and, (3) initiation of the hypothesized MOA is highly species-dependent, as activation of nuclear receptors relies upon interaction of a chemical with species-divergent ligand-binding domains (Jones *et al.*, 2000; Kretschmer *et al.*, 2005; LeCluyse, 2001; Timsit *et al.*, 2007). The proposed initiating key event in the hypothesized rat MOA presented an easily measured target across multiple species. The hypothesis tested in Chapter 5 was that TCS activates human and rat PXR and CAR resulting in potential up-regulation of hepatic catabolism of thyroid hormones and decreased serum T4 concentrations. Species-specific *in vitro* receptor reporter assays were employed to make an interspecies comparison. First, a full-length receptor reporter assays was used to demonstrate activation of human and rat PXR (Puracyp, Inc.). Second, a set of chimeric receptor-reporter assays was utilized to test ligand-binding only for rat CAR and PXR, all three human CAR splice variants and human PXR, and mouse CAR and PXR (INDIGO Biosciences). The results demonstrated an interesting pattern that challenged preconceived notions of the involvement of rat PXR. A previous report of activation of human PXR by TCS (Jacobs et al., 2005), and agreement with our findings of up-regulated markers of Cyp3a in the rat (Paul et al., 2010a), motivated the theory that rat PXR was involved in mediating the downstream effects of TCS *in vivo*. Both the full-length and chimeric receptor reporter assays confirmed that TCS is a moderate activator of human PXR. However, TCS was not a rat PXR agonist in either receptor-reporter assay. Intriguingly, TCS appeared to be an inverse agonist for rat CAR, similar to the response of PB and CITCO against rat CAR. The similarities for the pattern of effects between TCS and PB was important for hCAR as well, and the results for the hCAR splice variants were extremely surprising. The present work demonstrated that

TCS was (1) an inverse agonist for hCAR1, the constitutively-active, ligand-independent splice variant (Auerbach *et al.*, 2005; Chen *et al.*, 2010; Savkur *et al.*, 2003); (2) a potent agonist for hCAR2, the ligand-dependent splice variant with only one other known agonist (Auerbach *et al.*, 2007; DeKeyser *et al.*, 2009); and, (3) an agonist for hCAR3, a largely ligand-dependent splice variant (Auerbach et al., 2005; Chen et al., 2010). Finally, our finding that TCS activated hPXR and not rPXR was consistent with previous reports that demonstrate that PB is known to moderately activate human PXR in receptor reporter assays (Fery *et al.*, 2010; Luo *et al.*, 2002) with no activating effect on rat PXR in receptor reporter assays (Fery et al., 2010; Sinz *et al.*, 2007).

First, it is important to underscore that this pattern of effects for TCS and the three human CAR splice variants is the same as the pattern for PB, suggesting that TCS may be PB-like in its activation of CAR for both the human and the rat. Second, the activation of hCAR2 by TCS was indeed striking and unexpected. To date, only DEHP has been identified as an agonist of hCAR2 (DeKeyser *et al.*, 2009); the previously reporter maximal activation of hCAR2 in a receptor-reporter assay was 6-fold vehicle control at 10 µM (DeKeyser et al., 2009), whereas in the chimeric receptor reporter assays employed here, TCS activated hCAR2 4-, 5-, and 20-fold DMSO vehicle control at 10, 20, and 30 µM, respectively. What is truly novel about the aggregate receptor activation findings is the potential for different receptors to mediate the same downstream effects in two different species. Thus, Chapter 5 is the first report of TCS interaction with rCAR and hCAR splice variants and a lack of activation of rat PXR, and confirms a previous report of hPXR activation by TCS.

The collection of studies included here demonstrates that TCS induced a PB-like response *in vivo* for thyroid hormones and markers of hepatic catabolism, and *in vitro* at the

nuclear receptor level. But what is a PB-like response? *In vivo* PB exposure is known to up-regulate Cyp2b activity and expression (Honkakoski *et al.*, 1998a; Honkakoski *et al.*, 1998b; Sueyoshi *et al.*, 2001) as well as Cyp3a1 (Lecureux *et al.*, 2009); increase hepatic microsomal glucuronidation activity in rats toward T4 and T4 elimination (Hood et al., 2003; Hood et al., 2000; Lecureux et al., 2009; Liu et al., 1995; Qatanani *et al.*, 2005); and decrease T4 consistently with minor decreases on T3 (Barter *et al.*, 1994; Capen, 1994; Lecureux et al., 2009). PB effects on TSH are not as consistent, with reports of little to no effect on TSH concentrations (Lecureux *et al.*, 2009; Vansell et al., 2001), though some small increases (50-100%) have been observed (Liu et al., 1995) (Note: PTU-induced increases in TSH range from 4- to 5-fold control (O'Connor *et al.*, 2002)). *In vitro* rat hepatocyte models have confirmed these actions, and provided further support for PB activation of CAR; recently, *in vitro* human models have demonstrated that PB activation of CAR proceeds via an indirect mechanism that does not involve ligand binding, but rather results in protein kinase C-mediated dephosphorylation of threonine 38 of hCAR1 with subsequent translocation to the nucleus and transcriptional regulatory activity (Mutoh *et al.*, 2009). *In vitro* PB exposures in human hepatocytes and cell lines have further demonstrated activation of CYP2B6 by CAR in response to PB (Maglich *et al.*, 2003; Sueyoshi *et al.*, 1999; Wang *et al.*, 2003) and potential cross-talk between promoter elements enabling CAR-mediated induction of CYP3A (Faucette *et al.*, 2006; Faucette *et al.*, 2007). As PB is prescribed as an anti-epileptic, PB is one of the few thyroid disrupting chemicals that has been studied in humans and has demonstrated that the hypothesized MOA is plausible. PB decreased thyroid hormones in humans *in vivo* (Curran *et al.*, 1991) and increased expression and activity of UGT1A1 in human hepatocytes (Ritter *et al.*, 1999). The PB-like response exhibited by TCS implies that if the correct dose were achieved, TCS could initiate a similar cascade of events in humans.

Overall the data presented herein suggest that activation of nuclear receptors leading to increased hepatic catabolism of thyroid hormones and systemic decreases in T4 is plausible for the rat, following short-term and developmental exposures. The initiating key event, perhaps with a species-dependent bias for the nuclear receptor involved, occurs in an *in vitro* human model. Further, the pattern of effects observed for TCS and rat and human nuclear receptors suggested a PB-like response, and PB is one of the only chemicals for which the hypothesized MOA has actually been observed in humans. An obvious remaining question is: based on the dose-response observed in the rat, is it possible that TCS-induced hypothyroxinemia would occur in humans? While the MOA for TCS-induced hypothyroxinemia is plausible in humans, few data describing human exposure are available to compare to rats.

In a 14-day study of 12 adults, the participants were asked to brush their teeth for 3 min with 2 cm of Colgate Total ® toothpaste each day containing 0.3% TCS (Allmyr *et al.*, 2009). Measurement of TCS in the sera of these volunteers demonstrated that the median serum concentration was 54 ng/g (assuming a human plasma density of 1.025 ng/mL, this would be 55 ng/mL), with the maximum measured serum concentration of 303 ng/mL. Importantly, the volunteers in the human study experienced no effects on a marker of CYP3A4 activity and no change in serum free T4 concentrations (Allmyr *et al.*, 2009). The minimum serum concentration that elicited a significant effect on T4 in rats was 5120 ng/mL in PND22 dams, which corresponded to a statistically significant 15% decrease in serum T4. Comparison of this value with the highest measured human serum concentration yields a 17-fold margin of exposure. The lowest average serum concentration in rats that elicited a 30% decrease in T4, 19,000 ng/mL in PND22 dams, would yield a 60-fold margin of exposure. Comparisons of serum TCS concentrations and lowest observable effect levels for T4

decreases in rats to a human study that described no effects of TCS exposure on a marker of CYP3A4 activity of serum free T4 concentrations make conclusions difficult. The only conclusion that can therefore be derived from this comparison is that humans are not 17-fold or 60-fold more sensitive than rats to TCS exposure. Based on a comparison of serum concentrations of TCS across species, it appears unlikely that TCS exposure alone would cause hypothyroxinemia in humans.

Several uncertainties remain for the thyroid-disrupting activity of TCS specifically, nuclear receptor-mediated thyroid hormone homeostasis, and the function of TCS in the milieu of thyroid-disrupting chemicals to which the population is exposed. This dissertation research has only addressed one potential MOA for TCS-induced hypothyroxinemia. The contribution of other potential MOAs has not been assessed, and a poor correlation between UGT activity increases and T4 decreases *in viv*o may suggest the involvement of other MOAs or perhaps just other catabolic activities not fully considered here such as sulfotransferase activity. Future work would be required to understand the relative contribution of TCS activation of hCAR splice variants and hPXR; i.e., in a primary human hepatocyte, what is the overall resulting outcome of the potential interactions of TCS with multiple receptor types? Since hCAR2 is only 30% of the transcriptome (Auerbach et al., 2007), with hCAR1 comprising the majority of CAR transcripts in hepatocytes, does TCS activation of hCAR2 result in strong downstream up-regulation of CAR transcriptional targets? If TCS is a non-specific activator of both hPXR and hCAR, then to which receptor does TCS have the greatest affinity?

Further *in vivo* work with animals or *in vitro* work with nuclear receptor targets might help address the question of how TCS might contribute to the thyroid-disrupting activity of a mixture of chemicals. Since TCS is a low potency thyroid hormone disruptor, and exposure

concentrations in humans are low, is TCS a relevant component of the mixture to which humans are exposed? Clearly the most salient future experiment is exposure of human hepatocytes to demonstrate the impact of TCS exposure on markers of Phase I and Phase II metabolism in a more relevant human model.

A further consideration for extension of this work is the use of nuclear receptor reporters assays to screen for other toxicants like TCS that disrupt TH homeostasis via interaction with nuclear receptors and subsequent up-regulation in hepatic catabolism and elimination of THs. The use of PXR reporter assays is found commonly in the literature, generally as a means for identifying compounds that may lead to adverse drug interactions centered on CYP3A4-mediated metabolism (Fery et al., 2010; Luo et al., 2002; Shukla *et al.*, 2009; Shukla *et al.*, 2011; Yueh *et al.*, 2005), but the use of these assays as screening for potential thyrotoxicants is growing (Creusot *et al.*, 2010; Lemaire *et al.*, 2004; Rotroff *et al.*, 2010). To this end, following characterization of TCS alone in full-length PXR receptor-reporter assays, the method was extended to consider environmental water samples containing a complex and uncharacterized mixture of compounds (Appendix 7). This extended work underscores that comparative *in vitro* nuclear receptor reporter models may be used to evaluate the MOA for one compound, or to evaluate an environmental mixture for its potential thyroid-disupting activity via an MOA similar to the one proposed for TCS. Increasing use of nuclear receptor reporter assays for screening for thyroid-disrupting chemicals necessitates a greater understanding of the molecular events involved in nuclear receptor activation and nuclear receptor transcriptional regulation of downstream metabolism, e.g. co-activator/co-repressor recruitment, the involvement of multiple receptors and/or response elements, the contributions of multiple splice variants to downstream transcriptional changes, etc. Further research in this area using prototypical TH disruptors

may provide additional insight into molecular events that may uniquely distinguish subclasses of compounds that interact with nuclear receptors to increase hepatic catabolism and elimination of THs.

In summary, this research provides important findings for understanding the mode-of-action (MOA) for TCS-induced hypothyroxinemia in rats and provides initial support for the plausibility of this mode-of-action in a human model. Disruption of thyroid hormones by TCS and characterization of a MOA for these thyroid hormone changes following short-term and developmental exposures were previously unreported. The data presented within provide a critical metric for comparing exposures in rat studies to human exposures. Importantly, the initiating molecular key event of the proposed MOA for TCS-induced hypothyroxinemia was evaluated to demonstrate that nuclear receptor interactions with TCS occur in both rat and human models.

References

Adolfsson-Erici, M., M. Pettersson, J. Parkkonen and J. Sturve (2002). Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**, 1485-1489.

Allmyr, M., M. Adolfsson-Erici, M. S. McLachlan and G. Sandborgh-Englund (2006a). Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *The Science of the total environment* **372**, 87-93.

Allmyr, M., M. S. McLachlan, G. Sandborgh-Englund and M. Adolfsson-Erici (2006b). Determination of triclosan as its pentafluorobenzoyl ester in human plasma and milk using electron capture negative ionization mass spectrometry. *Analytical chemistry* **78**, 6542-6546.

Allmyr, M., F. Harden, L. M. Toms, J. F. Mueller, M. S. McLachlan, M. Adolfsson-Erici and G. Sandborgh-Englund (2008). The influence of age and gender on triclosan concentrations in Australian human blood serum. *The Science of the total environment* **393**, 162-167.

Allmyr, M., G. Panagiotidis, E. Sparve, U. Diczfalusy and G. Sandborgh-Englund (2009). Human exposure to triclosan via toothpaste does not change CYP3A4 activity or plasma concentrations of thyroid hormones. *Basic Clin Pharmacol Toxicol* **105**, 339-344.

Auerbach, S. S., M. A. Stoner, S. Su and C. J. Omiecinski (2005). Retinoid X receptor-alpha-dependent transactivation by a naturally occurring structural variant of human constitutive androstane receptor (NR1I3). *Molecular pharmacology* **68**, 1239-1253.

Auerbach, S. S., J. G. Dekeyser, M. A. Stoner and C. J. Omiecinski (2007). CAR2 displays unique ligand binding and RXRalpha heterodimerization characteristics. *Drug metabolism and disposition: the biological fate of chemicals* **35**, 428-439.

Axelstad, M., P. R. Hansen, J. Boberg, M. Bonnichsen, C. Nellemann, S. P. Lund, K. S. Hougaard and U. Hass (2008). Developmental neurotoxicity of propylthiouracil (PTU) in rats: relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes. *Toxicology and applied pharmacology* **232**, 1-13.

Barter, R. A. and C. D. Klaassen (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. *Toxicology and applied pharmacology* **128**, 9-17.

Berbel, P., J. L. Mestre, A. Santamaria, I. Palazon, A. Franco, M. Graells, A. Gonzalez-Torga and G. M. de Escobar (2009). Delayed neurobehavioral development in children born to pregnant women with mild hypothyroxinemia during the first month of gestation: the importance of early iodine supplementation. *Thyroid* **19**, 511-519.

Black, J. G., D. Howes and T. Rutherford (1975). Percutaneous absorption and metabolism of Irgasan DP300. *Toxicology* **3**, 33-47.

Brucker-Davis, F. (1998). Effects of environmental synthetic chemicals on thyroid function. *Thyroid* **8**, 827-856.

Capen, C. C. (1994). Mechanisms of chemical injury of thyroid gland. *Progress in clinical and biological research* **387**, 173-191.

Chen, T., L. M. Tompkins, L. Li, H. Li, G. Kim, Y. Zheng and H. Wang (2010). A single amino acid controls the functional switch of human constitutive androstane receptor (CAR) 1 to the xenobiotic-sensitive splicing variant CAR3. *The Journal of pharmacology and experimental therapeutics* **332**, 106-115.

Creusot, N., S. Kinani, P. Balaguer, N. Tapie, K. LeMenach, E. Maillot-Marechal, J. M. Porcher, H. Budzinski and S. Ait-Aissa (2010). Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: screening of chemicals and application to water samples. *Anal Bioanal Chem* **396**, 569-583.

Crofton, K. M., P. R. Kodavanti, E. C. Derr-Yellin, A. C. Casey and L. S. Kehn (2000). PCBs, thyroid hormones, and ototoxicity in rats: cross-fostering experiments demonstrate the impact of postnatal lactation exposure. *Toxicol Sci* **57**, 131-140.

Curran, P. G. and L. J. DeGroot (1991). The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. *Endocr Rev* **12**, 135-150.

Dayan, A. D. (2007). Risk assessment of triclosan [Irgasan((R))] in human breast milk. *Food Chem Toxicol* **45**, 125-129.

DeKeyser, J. G., M. C. Stagliano, S. S. Auerbach, K. S. Prabhu, A. D. Jones and C. J. Omiecinski (2009). Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant CAR2. *Molecular pharmacology* **75**, 1005-1013.

Faucette, S. R., T. Sueyoshi, C. M. Smith, M. Negishi, E. L. Lecluyse and H. Wang (2006). Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *The Journal of pharmacology and experimental therapeutics* **317**, 1200-1209.

Faucette, S. R., T. C. Zhang, R. Moore, T. Sueyoshi, C. J. Omiecinski, E. L. LeCluyse, M. Negishi and H. Wang (2007). Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *The Journal of pharmacology and experimental therapeutics* **320**, 72-80.

Fery, Y., S. O. Mueller and D. Schrenk (2010). Development of stably transfected human and rat hepatoma cell lines for the species-specific assessment of xenobiotic response enhancer module (XREM)-dependent induction of drug metabolism. *Toxicology* **277**, 11-19.

Gilbert, M. E. and L. Sui (2006). Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. *Brain Res* **1069**, 10-22.

Goldey, E. S., L. S. Kehn, G. L. Rehnberg and K. M. Crofton (1995). Effects of developmental hypothyroidism on auditory and motor function in the rat. *Toxicology and applied pharmacology* **135**, 67-76.

Goldey, E. S. and K. M. Crofton (1998). Thyroxine replacement attenuates hypothyroxinemia, hearing loss, and motor deficits following developmental exposure to Aroclor 1254 in rats. *Toxicol Sci* **45**, 94-105.

Haddow, J. E., G. E. Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein (1999). Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *The New England journal of medicine* **341**, 549-555.

Hanioka, N., H. Jinno, T. Nishimura and M. Ando (1997). Effect of 2,4,4'-trichloro-2'-hydroxydiphenyl ether on cytochrome P450 enzymes in the rat liver. *Chemosphere* **34**, 719-730.

Henrichs, J., J. J. Bongers-Schokking, J. J. Schenk, A. Ghassabian, H. G. Schmidt, T. J. Visser, H. Hooijkaas, S. M. de Muinck Keizer-Schrama, A. Hofman, V. V. Jaddoe, W. Visser, E. A. Steegers, F. C. Verhulst, Y. B. de Rijke and H. Tiemeier (2010). Maternal thyroid function during early pregnancy and cognitive functioning in early childhood: the generation R study. *J Clin Endocrinol Metab* **95**, 4227-4234.

Honkakoski, P. and M. Negishi (1998a). Regulatory DNA elements of phenobarbital-responsive cytochrome P450 CYP2B genes. *J Biochem Mol Toxicol* **12**, 3-9.

Honkakoski, P., I. Zelko, T. Sueyoshi and M. Negishi (1998b). The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18**, 5652-5658.

Hood, A., R. Hashmi and C. D. Klaassen (1999). Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. *Toxicology and applied pharmacology* **160**, 163-170.

Hood, A. and C. D. Klaassen (2000). Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. *Toxicol Sci* **55**, 78-84.

Hood, A., M. L. Allen, Y. Liu, J. Liu and C. D. Klaassen (2003). Induction of T(4) UDP-GT activity, serum thyroid stimulating hormone, and thyroid follicular cell proliferation in mice treated with microsomal enzyme inducers. *Toxicology and applied pharmacology* **188**, 6-13.

Jacobs, M. N., G. T. Nolan and S. R. Hood (2005). Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicology and applied pharmacology* **209**, 123-133.

Jinno, H., N. Hanioka, S. Onodera, T. Nishimura and M. Ando (1997). Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel. *Xenobiotica; the fate of foreign compounds in biological systems* **27**, 681-692.

Jones, S. A., L. B. Moore, J. L. Shenk, G. B. Wisely, G. A. Hamilton, D. D. McKee, N. C. Tomkinson, E. L. LeCluyse, M. H. Lambert, T. M. Willson, S. A. Kliewer and J. T. Moore (2000). The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Molecular endocrinology (Baltimore, Md* **14**, 27-39.

Kato, Y., H. Suzuki, S. Ikushiro, S. Yamada and M. Degawa (2005). Decrease in serum thyroxine level by phenobarbital in rats is not necessarily dependent on increase in hepatic UDP-glucuronosyltransferase. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1608-1612.

Kodavanti, P. R., C. G. Coburn, V. C. Moser, R. C. MacPhail, S. E. Fenton, T. E. Stoker, J. L. Rayner, K. Kannan and L. S. Birnbaum (2010). Developmental exposure to a commercial PBDE mixture, DE-71: neurobehavioral, hormonal, and reproductive effects. *Toxicol Sci* **116**, 297-312.

Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber and H. T. Buxton (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environmental science & technology* **36**, 1202-1211.

Kooistra, L., S. Crawford, A. L. van Baar, E. P. Brouwers and V. J. Pop (2006). Neonatal effects of maternal hypothyroxinemia during early pregnancy. *Pediatrics* **117**, 161-167.

Kretschmer, X. C. and W. S. Baldwin (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chemico-biological interactions* **155**, 111-128.

Lavado-Autric, R., E. Auso, J. V. Garcia-Velasco, C. Arufe Mdel, F. Escobar del Rey, P. Berbel and G. Morreale de Escobar (2003). Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. *The Journal of clinical investigation* **111**, 1073-1082.

LeCluyse, E. L. (2001). Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chemico-biological interactions* **134**, 283-289.

Lecureux, L., M. Z. Dieter, D. M. Nelson, L. Watson, H. Wong, B. Gemzik, C. D. Klaassen and L. D. Lehman-McKeeman (2009). Hepatobiliary disposition of thyroid hormone in Mrp2-deficient TR- rats: reduced biliary excretion of thyroxine glucuronide does not prevent xenobiotic-induced hypothyroidism. *Toxicol Sci* **108**, 482-491.

Lemaire, G., G. de Sousa and R. Rahmani (2004). A PXR reporter gene assay in a stable cell culture system: CYP3A4 and CYP2B6 induction by pesticides. *Biochem Pharmacol* **68**, 2347-2358.

Li, Y., Z. Shan, W. Teng, X. Yu, C. Fan, X. Teng, R. Guo, H. Wang, J. Li, Y. Chen, W. Wang, M. Chawinga, L. Zhang, L. Yang, Y. Zhao and T. Hua (2010). Abnormalities of maternal thyroid function during pregnancy affect neuropsychological development of their children at 25-30 months. *Clinical endocrinology* **72**, 825-829.

Liu, J., Y. Liu, R. A. Barter and C. D. Klaassen (1995). Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *The Journal of pharmacology and experimental therapeutics* **273**, 977-985.

Luo, G., M. Cunningham, S. Kim, T. Burn, J. Lin, M. Sinz, G. Hamilton, C. Rizzo, S. Jolley, D. Gilbert, A. Downey, D. Mudra, R. Graham, K. Carroll, J. Xie, A. Madan, A. Parkinson, D. Christ, B. Selling, E. LeCluyse and L. S. Gan (2002). CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **30**, 795-804.

Maglich, J. M., D. J. Parks, L. B. Moore, J. L. Collins, B. Goodwin, A. N. Billin, C. A. Stoltz, S. A. Kliewer, M. H. Lambert, T. M. Willson and J. T. Moore (2003). Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* **278**, 17277-17283.

Morse, D. C., D. Groen, M. Veerman, C. J. van Amerongen, H. B. Koeter, A. E. Smits van Prooije, T. J. Visser, J. H. Koeman and A. Brouwer (1993). Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. *Toxicology and applied pharmacology* **122**, 27-33.

Mutoh, S., M. Osabe, K. Inoue, R. Moore, L. Pedersen, L. Perera, Y. Rebolloso, T. Sueyoshi and M. Negishi (2009). Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). *J Biol Chem* **284**, 34785-34792.

O'Connor, J. C., S. R. Frame and G. S. Ladics (2002). Evaluation of a 15-day screening assay using intact male rats for identifying steroid biosynthesis inhibitors and thyroid modulators. *Toxicol Sci* **69**, 79-91.

Opazo, M. C., A. Gianini, F. Pancetti, G. Azkcona, L. Alarcon, R. Lizana, V. Noches, P. A. Gonzalez, M. P. Marassi, S. Mora, D. Rosenthal, E. Eugenin, D. Naranjo, S. M. Bueno, A. M. Kalergis and C. A. Riedel (2008). Maternal hypothyroxinemia impairs spatial learning and synaptic nature and function in the offspring. *Endocrinology* **149**, 5097-5106.

Paul, K. B., J. M. Hedge, M. J. DeVito and K. M. Crofton (2010a). Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol Sci* **113**, 367-379.

Paul, K. B., J. M. Hedge, M. J. Devito and K. M. Crofton (2010b). Developmental triclosan exposure decreases maternal and neonatal thyroxine in rats. *Environmental toxicology and chemistry / SETAC* **29**, 2840-2844.

Pop, V. J., J. L. Kuijpens, A. L. van Baar, G. Verkerk, M. M. van Son, J. J. de Vijlder, T. Vulsma, W. M. Wiersinga, H. A. Drexhage and H. L. Vader (1999). Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clinical endocrinology* **50**, 149-155.

Pop, V. J., E. P. Brouwers, H. L. Vader, T. Vulsma, A. L. van Baar and J. J. de Vijlder (2003). Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clinical endocrinology* **59**, 282-288.

Qatanani, M., J. Zhang and D. D. Moore (2005). Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. *Endocrinology* **146**, 995-1002.

Richardson, T. A. and C. D. Klaassen (2010a). Role of UDP-glucuronosyltransferase (UGT) 2B2 in metabolism of triiodothyronine: effect of microsomal enzyme inducers in Sprague Dawley and UGT2B2-deficient Fischer 344 rats. *Toxicol Sci* **116**, 413-421.

Richardson, T. A. and C. D. Klaassen (2010b). Disruption of thyroid hormone homeostasis in Ugt1a-deficient Gunn rats by microsomal enzyme inducers is not due to enhanced thyroxine glucuronidation. *Toxicology and applied pharmacology* **248**, 38-44.

Ritter, J. K., F. K. Kessler, M. T. Thompson, A. D. Grove, D. J. Auyeung and R. A. Fisher (1999). Expression and inducibility of the human bilirubin UDP-glucuronosyltransferase UGT1A1 in liver and cultured primary hepatocytes: evidence for both genetic and environmental influences. *Hepatology* **30**, 476-484.

Rodricks, J. V., J. A. Swenberg, J. F. Borzelleca, R. R. Maronpot and A. M. Shipp (2010). Triclosan: a critical review of the experimental data and development of margins of safety for consumer products. *Critical reviews in toxicology* **40**, 422-484.

Rotroff, D. M., A. L. Beam, D. J. Dix, A. Farmer, K. M. Freeman, K. A. Houck, R. S. Judson, E. L. LeCluyse, M. T. Martin, D. M. Reif and S. S. Ferguson (2010). Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by ToxCast chemicals. *J Toxicol Environ Health B Crit Rev* **13**, 329-346.

Royland, J. E., J. S. Parker and M. E. Gilbert (2008). A genomic analysis of subclinical hypothyroidism in hippocampus and neocortex of the developing rat brain. *Journal of neuroendocrinology* **20**, 1319-1338.

Sandborgh-Englund, G., M. Adolfsson-Erici, G. Odham and J. Ekstrand (2006). Pharmacokinetics of triclosan following oral ingestion in humans. *Journal of toxicology and environmental health* **69**, 1861-1873.

Savkur, R. S., Y. Wu, K. S. Bramlett, M. Wang, S. Yao, D. Perkins, M. Totten, G. Searfoss, 3rd, T. P. Ryan, E. W. Su and T. P. Burris (2003). Alternative splicing within the ligand binding domain of the human constitutive androstane receptor. *Mol Genet Metab* **80**, 216-226.

Sharlin, D. S., R. Bansal and R. T. Zoeller (2006). Polychlorinated biphenyls exert selective effects on cellular composition of white matter in a manner inconsistent with thyroid hormone insufficiency. *Endocrinology* **147**, 846-858.

Shukla, S. J., D. T. Nguyen, R. Macarthur, A. Simeonov, W. J. Frazee, T. M. Hallis, B. D. Marks, U. Singh, H. C. Eliason, J. Printen, C. P. Austin, J. Inglese and D. S. Auld (2009). Identification of pregnane X receptor ligands using time-resolved fluorescence resonance energy transfer and quantitative high-throughput screening. *Assay Drug Dev Technol* **7**, 143-169.

Shukla, S. J., S. Sakamuru, R. Huang, T. A. Moeller, P. Shinn, D. Vanleer, D. S. Auld, C. P. Austin and M. Xia (2011). Identification of clinically used drugs that activate pregnane X receptors. *Drug metabolism and disposition: the biological fate of chemicals* **39**, 151-159.

Siddiqui, W. H. and H. S. Buttar (1979). Pharmacokinetics of triclosan in rat after intravenous and intravaginal administration. *Journal of environmental pathology and toxicology* **2**, 861-871.

Sinz, M. W., D. Pray and J. Raucy (2007). The utility of stable cell lines to assess species differences in PXR transactivation. *Drug Metab Lett* **1**, 147-152.

Sueyoshi, T., T. Kawamoto, I. Zelko, P. Honkakoski and M. Negishi (1999). The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274**, 6043-6046.

Sueyoshi, T. and M. Negishi (2001). Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* **41**, 123-143.

Timsit, Y. E. and M. Negishi (2007). CAR and PXR: the xenobiotic-sensing receptors. *Steroids* **72**, 231-246.

Vansell, N. R. and C. D. Klaassen (2001). Increased biliary excretion of thyroxine by microsomal enzyme inducers. *Toxicology and applied pharmacology* **176**, 187-194.

Veldhoen, N., R. C. Skirrow, H. Osachoff, H. Wigmore, D. J. Clapson, M. P. Gunderson, G. Van Aggelen and C. C. Helbing (2006). The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquatic toxicology (Amsterdam, Netherlands)* **80**, 217-227.

Wang, H., S. Faucette, T. Sueyoshi, R. Moore, S. Ferguson, M. Negishi and E. L. LeCluyse (2003). A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* **278**, 14146-14152.

Wang, L. Q., C. N. Falany and M. O. James (2004). Triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 1162-1169.

Wang, L. Q. and M. O. James (2006). Inhibition of sulfotransferases by xenobiotics. *Current drug metabolism* **7**, 83-104.

Yueh, M. F., M. Kawahara and J. Raucy (2005). High volume bioassays to assess CYP3A4-mediated drug interactions: induction and inhibition in a single cell line. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 38-48.

Zhou, T., D. G. Ross, M. J. DeVito and K. M. Crofton (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zhou, T., M. M. Taylor, M. J. DeVito and K. M. Crofton (2002). Developmental exposure to brominated diphenyl ethers results in thyroid hormone disruption. *Toxicol Sci* **66**, 105-116.

Appendix 1:
Short-Term in Vivo Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine

Kevin M. Crofton[1], Katie B. Paul[2], Michael J. DeVito[3] and Joan M. Hedge[1]

[1] Neurotoxicology Division and [3]Experimental Toxicology Division,
National Health and Environmental Effects Research Laboratory,
Office of Research and Development, U.S. EPA, Research Triangle Park, NC;
[2]Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC.

Correspondence: Kevin M. Crofton, Ph.D., Neurotoxicology Division, MD-B105-04, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; phone: 919-541-2672; email: crofton.kevin@epa.gov

1. Abstract

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a chlorinated phenolic antibacterial compound found as an active ingredient in many personal care and household products. The structural similarity of triclosan to thyroid hormones and recent studies demonstrating activation of the human pregnane X receptor (PXR) and inhibition of diiodothyronine (T_2) sulfotransferases, have raised concerns about adverse effects on thyroid homeostasis. The current research tested the hypothesis that triclosan alters circulating concentrations of thyroxine. The hypothesis was tested using a four-day oral triclosan exposure (0–1000 mg/kg/day) in weanling female Long Evans rats, followed by measurement of circulating levels of serum total thyroxine (T_4). Dose-dependent decreases in total T_4 were observed. The benchmark-dose (BMD) and lower bound on the BMD (BMDL) for the effects on T_4 were 69.7 and 35.6 mg/kg/day, respectively. These data demonstrate that triclosan disrupts thyroid hormone homeostasis in rats.

2. Introduction

Triclosan is a chlorinated phenolic antibacterial compound used as an active ingredient in many personal care and household products (Bhargava *et al.*, 1996; Dayan, 2007). The bactericidal activity of triclosan results from inhibition of the enoyl-acyl carrier protein reductase (ENR) of gram-negative and gram-positive bacteria, thereby preventing bacterial lipid biosynthesis(Heath *et al.*, 2000; Levy *et al.*, 1999; McMurry *et al.*, 1998). The ubiquitous usage in consumer products has led to widespread environmental contamination evidenced by detection of triclosan in wastewater effluent in the US, UK, Japan, and other countries (Kanda *et al.*, 2003; Loraine *et al.*, 2006; Nakada *et al.*, 2006). Triclosan has been found in fish exposed to wastewater treatment plant effluents (Adolfsson-Erici *et al.*, 2002),

in human breast milk samples (Adolfsson-Erici et al., 2002; Dayan, 2007), and in urine samples from young girls (Wolff *et al.*, 2007).

The structural similarity to thyroid hormones (THs, Figure 1) and evidence for human exposures have increased concern about possible endocrine disrupting effects of triclosan (Veldhoen *et al.*, 2006). Foran et al. (2000) studied the potential estrogenic effects of triclosan in *Oryzias latipes* (medaka) and concluded that it is not estrogenic, but may be a weak androgen. Vitellogenin induction, a biomarker for endocrine activity, has been found in medaka and *Xenpous laevis* (clawed frog) and was attributed to weak estrogenic activity of a triclosan metabolite (Ishibashi *et al.*, 2004). There is also evidence that triclosan may disrupt thyroid mediated development. Exposure of *Rana catesbeiana* (North American bullfrog) to triclosan resulted in accelerated thyroid hormone dependent metamorphosis and decreased expression of thyroid receptor beta mRNA (Veldhoen et al., 2006). Triclosan also affects a number of biochemical processes important for thyroid hormone homeostasis. Triclosan induces hepatic EROD and PROD activity, and cytochromes P450 2B1/2 (Hanioka *et al.*, 1996; Jinno *et al.*, 1997). Inhibition of iodothyronine sulfotransferase activity was demonstrated in both rat and human hepatic microsomes (Schuur *et al.*, 1998; Wang *et al.*, 2004). Furthermore, triclosan activates pregnane-X receptor(PXR)-mediated transcription in transient transfection studies with human PXR in a human hepatoma cell line (Jacobs *et al.*, 2005). PXR activation leads to increases in Phase I and II hepatic biotransformation enzymes, including glucuronidases that catabolize thyroid hormones (THs) (You, 2004). These enzymes are important in maintaining euthyroid hormone concentrations (Capen, 1994; DeVito *et al.*, 1999; Hill *et al.*, 1998). Developmental disruption of circulating THs concentrations leads to adverse outcomes including altered neurodevelopment (Koibuchi *et*

al., 2006; Mitchell *et al.*, 2004; Morreale de Escobar *et al.*, 2004; Zoeller *et al.*, 2000). Currently it is unknown whether triclosan affects circulating concentrations of thyroid hormones.

The present work tested the hypothesis that *in vivo* triclosan exposure alters circulating levels of thyroxine (T4). T4 has been shown to be a sensitive biomarker for disruption of thyroid homeostasis in rats (DeVito et al., 1999). A four-day exposure to rats was employed as this protocol has been previously used to detect thyroid disrupting chemicals (Craft *et al.*, 2002; Crofton, 2004; Zhou *et al.*, 2001). In addition, triclosan up-regulated rat hepatic microsomal enzymes after a four-day exposure (Hanioka et al., 1996).

3. Materials and Methods

Animals. Long-Evans female rats at 21-23 days of age were obtained from Charles River Laboratories Inc. (Raleigh, NC). These 80 rats were allowed 2-4 days of acclimation in an American Association for Accreditation of Laboratory Animal Care (AALAC)-approved animal facility prior to treatment. Animals were housed two per standard plastic hanging cage (45 cm x 24 cm x 20 cm), with heat sterilized pine shavings bedding (Northeastern Products Corp., Warrenburg, NY). Colony rooms were maintained at 21±2 ^{o}C with 50±10% humidity on a photo-period of 12L:12D (0600 – 1800 hr). Food (Purina Rodent Chow #5001, Barnes Supply Co., Durham, NC) and tap water were provided *ad libitum*. All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the US EPA.

Chemicals. Triclosan (5-Chloro-2-(2,4-dichloro-phenoxy)phenol, 99.6%, CAS#3380-34-5, Lot#06415CD) was obtained from Aldrich Chemical Company (St Louis, Mo., Cat#524190-10G). The dosing solutions at concentrations of 0, 10, 30, 100, 300 and 1000mg/kg/day were

prepared by mixing triclosan with corn oil and sonicating for 30 min at room temperature. The 1000 mg/kg/day dose precipitated within 12 hrs, therefore, it was sonicated daily before use. Dosing volume was 1.0 ml corn oil/kg body weight. Rats at 27-29 days of age were exposed via oral gavage for four consecutive days (n=16 for all groups except 10 and 1000 mg/kg/day where n=8/group). All dosing was conducted between 0900 and 1000 hrs. Young female rats were chosen to be consistent with previous work in our lab using the short-term dosing model (Craft et al., 2002; Crofton, 2004; Zhou et al., 2001). Rats were randomly assigned to treatment groups by counter-balancing body weights. Body weights were recorded and dosing volumes adjusted daily. Approximately 24 hours after the final treatment rats (0900 – 1100 hrs) were moved into a holding room. Prior to termination, rats were weighed, acclimated for 30 min, and then taken to a separate room with a separate air supply and killed by decapitation. Trunk blood was collected, after decapitation, into serum separator tubes (Becton Dickinson, 36-6154). Serum was obtained after clotting whole blood for 30 min on ice, followed by centrifugation at 1278x g at 4^{o} C for 30 min and stored at -80^{o} C until analysis. Livers were weighed, quick frozen in liquid nitrogen and stored at -80^{o} C for future analyses.

Thyroxine assay. Serum total T4 was measured in duplicate by standard solid-phase Coat-A Count radioimmunoassay (RIA) kits (Siemens Medical Solutions Diagnostics, formerly Diagnostic Products Corporation, Los Angeles, CA). Intra-assay coefficient of variation for the triclosan data was 6.51%. The inter-assay coefficient-of-variance for six separate assays over a one year period of time was 7.68%.

Data analysis. All data were analyzed as ng T4/ml serum. The mean (±SE) for the control group is listed in the figure legend. Statistical significance was tested with a one-way

ANOVA followed by Duncan's Multiple Range Test, with acceptable significance level set at $p > 0.05$. All data are represented as mean ± SE (n=16 for 0, 30, 100, and 300 mg/kg/day doses and n=8 for 10 mg/kg/day and 1000mg/kg/day doses). No-observed-effects-levels (NOELs) were defined as the lowest dose without a significant effect.

Benchmark dose (BMD) and lower-bound confidence limit (BMDL) estimates were determined for alterations in serum thyroxine concentrations using USEPA Benchmark Dose Software (BMDS Version 1.40d). The Hill model was chosen to fit these continuous data according to the following equations:

$$y(x) = e_0 - \frac{(e_{max} * x^n)}{(b^n + x^n)}$$

where y is the response; x is the dose; e_0 is the estimated background response level; e_{max} is the maximal increase or decrease from background; b is the ED_{50}; n is the shape parameter. The benchmark response was set at a 20% decrease in T_4 concentration (Zhou et al., 2001). The BMDs were calculated from the Hill model fits to the data. The BMDL (lower-bound confidence limit) was calculated as the 95% lower confidence interval.

4. Results and Discussion

No clinical signs of toxicity were observed in any animals following the 4-day oral triclosan exposure. No treatment-related effects on body weight gain were found [$F(5, 74) = 0.40$, $p > 0.8443$], Body weight gains were between 26 and 28 g for all treatments. There was a significant increase in liver weight [$F(5,74) = 4.23$, $p > 0.002$] of 15% in the 1000 mg/kg group (Table 1). No other treatments were different than control.

Serum thyroxine concentrations were reduced in a dose dependent manner (Figure 2). Serum T4 was decreased 28, 34 and 53% following treatment with 100, 300, and 1000 mg/kg/day triclosan, respectively. This shows a significant main effect of treatment [$F(5, 74)=18.32$, $p>0.0001$]. The NOEL for triclosan was 30 mg/kg/day. The goodness-of-fit p-value for the Hill model was 0.2232. The BMD was 69.7 mg/kg/day and the BMDL was 35.6 mg/kg/day.

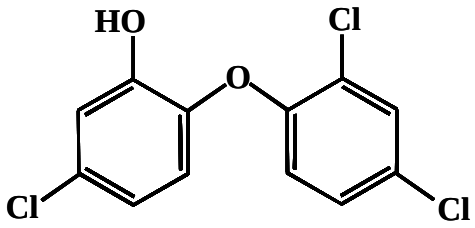
These data clearly demonstrate for the first time that triclosan decreases circulating concentrations of T_4 in rats. The mechanisms by which triclosan reduces serum T_4 are presently unknown, however, previous research suggests more than one possible mechanism. Evidence that PXR is activated by triclosan (Jacobs et al., 2005) suggests that decreases in T_4 may result from increases in the sulfonation or glucuronidation activity (Visser, 1996) via PXR-linked genes (Kretschmer *et al.*, 2005). This hypothesis is consistent with triclosan-induced up-regulation of P450 2B isozymes (Hanioka et al., 1996; Jinno et al., 1997). Activation of PXR is known to result in induction of cytochrome P450s and uridine diphosphoglucuronyl transferases (UGTs). UGTs are a family of isozymes, some of which glucuronidate THs, with subsequent excretion of the TH-glucuronide into the bile (Chen *et al.*, 2003; Mackenzie *et al.*, 2005; Zhou *et al.*, 2005). This is not consistent with a report that triclosan is a weak inhibitor of acetaminophen and bisphenol A glucuronidation (Wang et al., 2004). However, morphine glucuronidation was not affected, suggesting an isoform-specific inhibition (Wang et al., 2004). PXR activation has also been shown to regulate hepatic transporters (Klaassen *et al.*, 2005). These transporters can increase influx of T_4 into the liver, and efflux of free and glucuronidated T_4 into the bile (Friesema *et al.*, 2005). A combination of mechanisms may explain changes in TH levels, specifically the dose-

dependent decrease in T_4 observed in this study. Future research on triclosan should characterize its effects on thyroid homeostasis and the relevance of these findings to humans.

5. Acknowledgements

The authors would like to thank Drs. Douglas Wolf and Joyce Royland for comments on a previous version of this manuscript. K. Paul was supported by a grant from NIEHS, Research Grant T32-ES07126 with the Curriculum in Toxicology, University of North Carolina at Chapel Hill.

Triclosan



Thyroxine (T4)

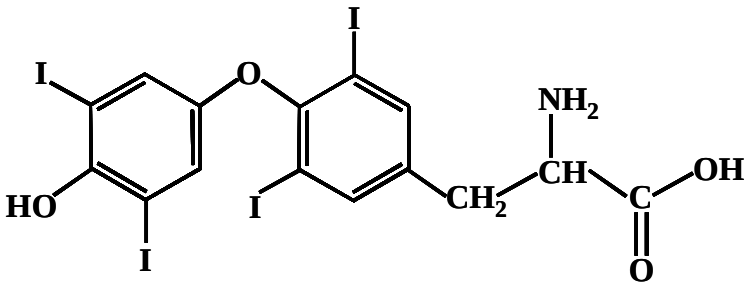


Figure A1.1. The structures of triclosan and thyroxine. Both chemicals are halogenated biphenyl ethers.

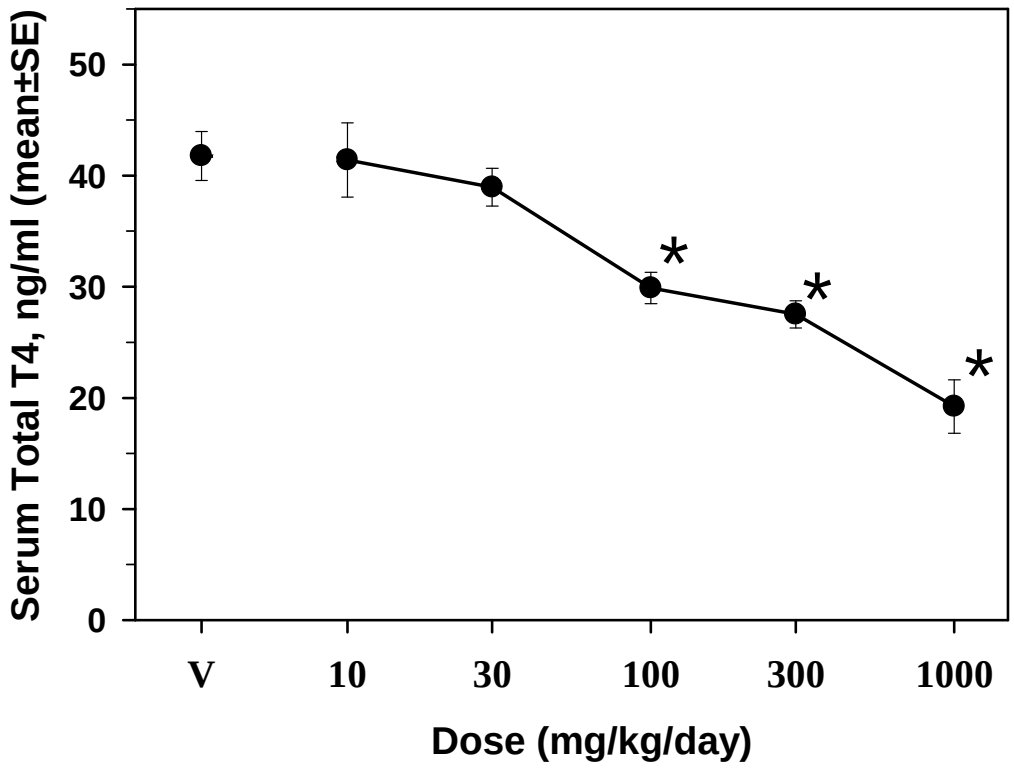


Figure A1.2. Short-term oral exposure to triclosan decreased serum total T4 concentrations. Data are expressed as group mean values (±SE), V = corn oil vehicle only control. Absolute T4 group mean value for the control group (0 mg/kg/day triclosan) is 41.8± 2.2 ng total T4/ml serum. [*Significantly different from the respective control ($p < 0.05$); n=16 for all groups except 10 and 1000 mg/kg/day where n=8/group]

Triclosan Dose (mg/kg)	N	Body Weight Gain[a], g (mean±SE)	Liver Weight, g (mean±SE)	Liver-Body Weight Ratio[b] (mean±SE)
0 (vehicle)	16	26.6 ± 0.73	4.85 ± 0.18	0.050 ± 0.0006
10	8	26.1 ± 1.05	4.46 ± 0.26	0.053 ±0.0007
30	16	28.5 ± 0.99	4.97 ±0.23	0.051 ±0.0005
100	16	26.7 ± 1.18	4.75 ± 0.25	0.050 ± 0.0004
300	16	27.2 ± 2.01	5.04 ± 0.26	0.052 ± 0.0006
1000	8	28.0 ± 1.31	6.26 ± 0.14*	0.056 ± 0.0008*

Notes: [a] Weight gain between Day 1 and Day 5.
[b] Absolute liver weight divided by absolute body weight.
* Significantly different from vehicle control group, $p<0.05$

Table A-1. Doses, group sizes, body weights, and liver weights.

References

Adolfsson-Erici, M., M. Pettersson, J. Parkkonen and J. Sturve (2002). Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**, 1485-1489.

Bhargava, H. N. and P. A. Leonard (1996). Triclosan: applications and safety. *American journal of infection control* **24**, 209-218.

Capen, C. C. (1994). Mechanisms of chemical injury of thyroid gland. *Progress in clinical and biological research* **387**, 173-191.

Chen, C., J. L. Staudinger and C. D. Klaassen (2003). Nuclear receptor, pregname X receptor, is required for induction of UDP-glucuronosyltranferases in mouse liver by pregnenolone-16 alpha-carbonitrile. *Drug metabolism and disposition: the biological fate of chemicals* **31**, 908-915.

Craft, E. S., M. J. DeVito and K. M. Crofton (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicol Sci* **68**, 372-380.

Crofton, K. M. (2004). Developmental disruption of thyroid hormone: correlations with hearing dysfunction in rats. *Risk Anal* **24**, 1665-1671.

Dayan, A. D. (2007). Risk assessment of triclosan [Irgasan((R))] in human breast milk. *Food Chem Toxicol* **45**, 125-129.

DeVito, M., L. Biegel, A. Brouwer, S. Brown, F. Brucker-Davis, A. O. Cheek, R. Christensen, T. Colborn, P. Cooke, J. Crissman, K. Crofton, D. Doerge, E. Gray, P. Hauser, P. Hurley, M. Kohn, J. Lazar, S. McMaster, M. McClain, E. McConnell, C. Meier, R. Miller, J. Tietge and R. Tyl (1999). Screening methods for thyroid hormone disruptors. *Environmental health perspectives* **107**, 407-415.

Friesema, E. C., J. Jansen, C. Milici and T. J. Visser (2005). Thyroid hormone transporters. *Vitamins and hormones* **70**, 137-167.

Hanioka, N., E. Omae, T. Nishimura, H. Jinno, S. Onodera, R. Yoda and M. Ando (1996). Interaction of 2,4,4'-trichloro-2'-hydroxydiphenyl ether with microsomal cytochrome P450-dependent monooxygenases in rat liver. *Chemosphere* **33**, 265-276.

Heath, R. J. and C. O. Rock (2000). A triclosan-resistant bacterial enzyme. *Nature* **406**, 145-146.

Hill, R. N., T. M. Crisp, P. M. Hurley, S. L. Rosenthal and D. V. Singh (1998). Risk assessment of thyroid follicular cell tumors. *Environmental health perspectives* **106**, 447-457.

Ishibashi, H., N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao and K. Arizono (2004). Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. *Aquatic toxicology (Amsterdam, Netherlands)* **67**, 167-179.

Jacobs, M. N., G. T. Nolan and S. R. Hood (2005). Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicology and applied pharmacology* **209**, 123-133.

Jinno, H., N. Hanioka, S. Onodera, T. Nishimura and M. Ando (1997). Irgasan DP 300 (5-chloro-2-(2,4-dichlorophenoxy)-phenol) induces cytochrome P450s and inhibits haem biosynthesis in rat hepatocytes cultured on Matrigel. *Xenobiotica; the fate of foreign compounds in biological systems* **27**, 681-692.

Kanda, R., P. Griffin, H. A. James and J. Fothergill (2003). Pharmaceutical and personal care products in sewage treatment works. *J Environ Monit* **5**, 823-830.

Klaassen, C. D. and A. L. Slitt (2005). Regulation of hepatic transporters by xenobiotic receptors. *Current drug metabolism* **6**, 309-328.

Koibuchi, N. and T. Iwasaki (2006). Regulation of brain development by thyroid hormone and its modulation by environmental chemicals. *Endocrine journal* **53**, 295-303.

Kretschmer, X. C. and W. S. Baldwin (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chemico-biological interactions* **155**, 111-128.

Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice and J. B. Rafferty (1999). Molecular basis of triclosan activity. *Nature* **398**, 383-384.

Loraine, G. A. and M. E. Pettigrove (2006). Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. *Environmental science & technology* **40**, 687-695.

Mackenzie, P. I., K. Walter Bock, B. Burchell, C. Guillemette, S. Ikushiro, T. Iyanagi, J. O. Miners, I. S. Owens and D. W. Nebert (2005). Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenetics and genomics* **15**, 677-685.

McMurry, L. M., M. Oethinger and S. B. Levy (1998). Triclosan targets lipid synthesis. *Nature* **394**, 531-532.

Mitchell, M. L. and R. Z. Klein (2004). The sequelae of untreated maternal hypothyroidism. *European journal of endocrinology / European Federation of Endocrine Societies* **151 Suppl 3**, U45-48.

Morreale de Escobar, G., M. J. Obregon and F. Escobar del Rey (2004). Role of thyroid hormone during early brain development. *European journal of endocrinology / European Federation of Endocrine Societies* **151 Suppl 3**, U25-37.

Nakada, N., T. Tanishima, H. Shinohara, K. Kiri and H. Takada (2006). Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. *Water research* **40**, 3297-3303.

Schuur, A. G., F. F. Legger, M. E. van Meeteren, M. J. Moonen, I. van Leeuwen-Bol, A. Bergman, T. J. Visser and A. Brouwer (1998). In vitro inhibition of thyroid hormone sulfation by hydroxylated metabolites of halogenated aromatic hydrocarbons. *Chemical research in toxicology* **11**, 1075-1081.

Veldhoen, N., R. C. Skirrow, H. Osachoff, H. Wigmore, D. J. Clapson, M. P. Gunderson, G. Van Aggelen and C. C. Helbing (2006). The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquatic toxicology (Amsterdam, Netherlands)* **80**, 217-227.

Visser, T. J. (1996). Pathways of thyroid hormone metabolism. *Acta medica Austriaca* **23**, 10-16.

Wang, L. Q., C. N. Falany and M. O. James (2004). Triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 1162-1169.

Wolff, M. S., S. L. Teitelbaum, G. Windham, S. M. Pinney, J. A. Britton, C. Chelimo, J. Godbold, F. Biro, L. H. Kushi, C. M. Pfeiffer and A. M. Calafat (2007). Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. *Environmental health perspectives* **115**, 116-121.

You, L. (2004). Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chemico-biological interactions* **147**, 233-246.

Zhou, J., J. Zhang and W. Xie (2005). Xenobiotic nuclear receptor-mediated regulation of UDP-glucuronosyl-transferases. *Current drug metabolism* **6**, 289-298.

Zhou, T., D. G. Ross, M. J. DeVito and K. M. Crofton (2001). Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol Sci* **61**, 76-82.

Zoeller, R. T. and K. M. Crofton (2000). Thyroid hormone action in fetal brain development and potential for disruption by environmental chemicals. *Neurotoxicology* **21**, 935-945.

Appendix 2: Supplemental Data Table for Chapter 2

Serum assays: Thyroxine (T4) data

Endpoint	Dose (mg/kg/d)	N per dose	Mean (ng T4/mL serum)	Standard deviation (SD) for ng T4/mL serum
T4	0	24	44.97	9.31
T4	10	8	41.41	9.46
T4	30	24	40.99	7.32
T4	100	24	33.24	7.35
T4	300	24	29.06	5.35
T4	1000	16	19.33	5.39

Serum assays: Triiodothyronine (T3) data

Endpoint	Dose (mg/kg/d)	N per dose	Mean (ng T3/dL serum)	Standard deviation (SD) for ng T3/dL serum
T3	0	8	107.45	11.73
T3	30	8	108.84	8.93
T3	100	8	98.61	5.96
T3	300	8	95.31	9.46
T3	1000	8	81.14	6.84

Serum assays: Thyroid-stimulating hormone (TSH) data

Endpoint	Dose (mg/kg/d)	N per dose	Mean (ng TSH/mL serum)	Standard deviation (SD) for ng TSH/mL serum
TSH	0	8	2.48	0.78
TSH	30	8	1.94	0.64
TSH	100	8	3.08	0.80
TSH	300	8	2.70	1.48
TSH	1000	8	3.12	1.87

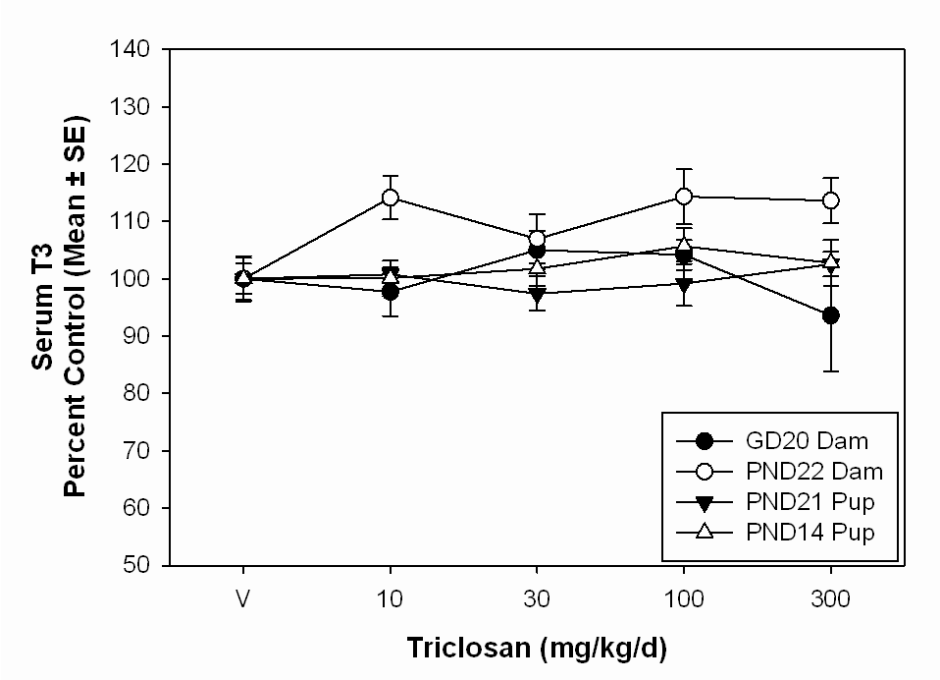
Appendix 3: Supplemental Data for Chapter 3

Thyroxine (T4) n and mean ± SE for each age and dose.

	Offspring								**Dams**		
	PND4			PND14			PND21		PND22		
Dose (mg/kg/day)	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
V	9	9.63	0.49	10	48.4	2.2	10	38.9	2.0	10	49.6	2.2
30	9	9.12	0.66	10	47.1	2.5	10	38.9	1.3	10	45.4	3.7
100	8	7.87	0.63	8	42.0	2.0	8	38.8	2.4	8	45.8	2.4
300	8	7.08	0.64	8	40.6	2.3	8	36.0	1.8	8	34.1	3.7

Appendix 4: Supplemental Data for Chapter 4

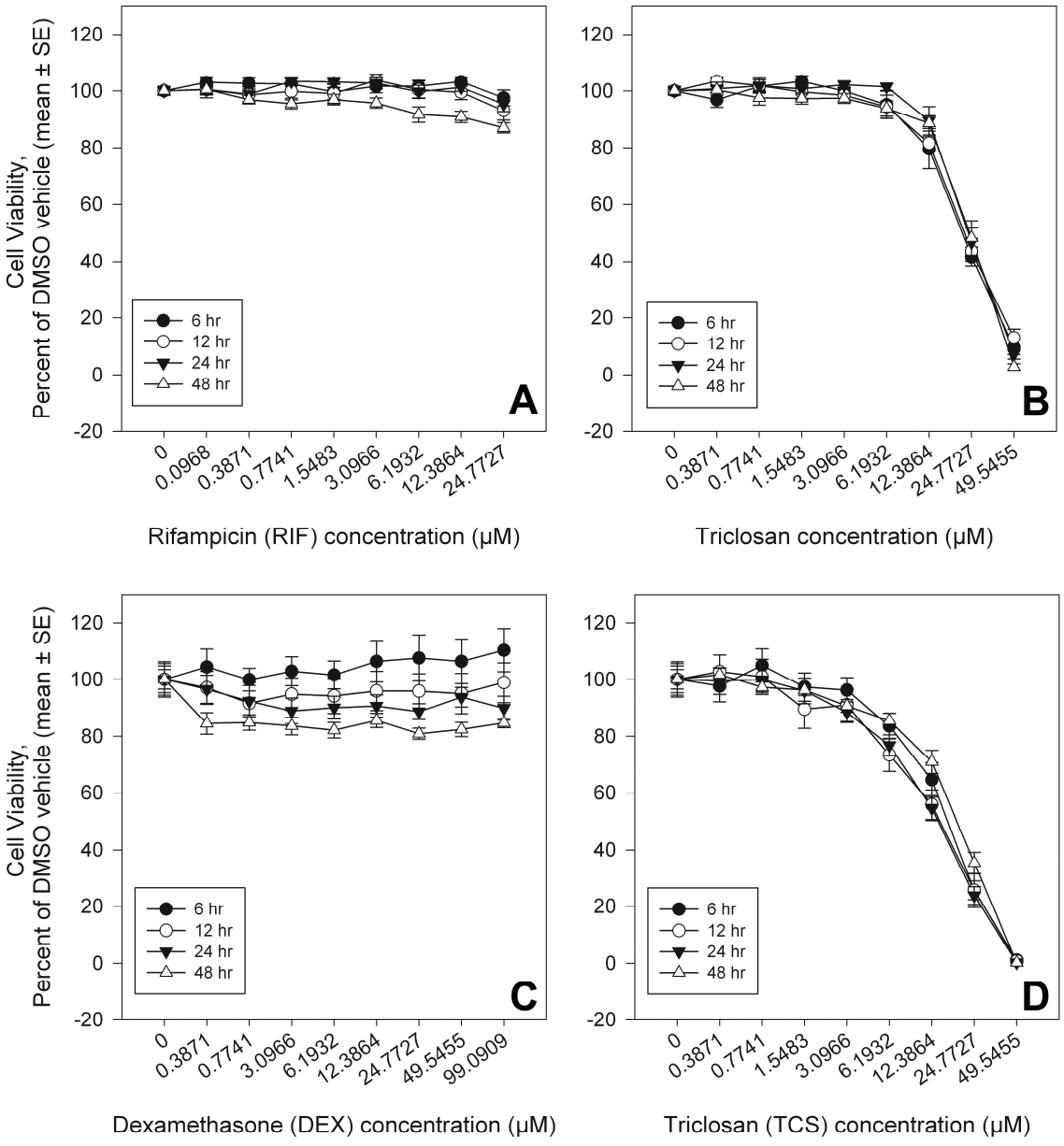
Triiodothyronine (T3) Concentrations in Dams and Offspring Following Perinatal TCS Exposure



Percent of control serum T3 for dams and PND14 and PND21 Offspring. GD20 and PND22 Dams had vehicle control values = 85.3 ± 3.1 and 70.4 ± 2.8 ng/dL, respectively. PND14 and PND21 pups had vehicle control values = 80.7 ±2.1 and 95.1 ± 3.5 ng/dL, respectively. V=vehicle control.

Appendix 5: Supplementary Data for Chapter 5

Time-course of Triclosan-Induced Cytotoxicity in DPX-2 and RPXR Receptor Reporter Cell Lines



Cytotoxicity of chemical treatments at 6, 12, 24, and 48 hr of chemical exposure. A. Effects of DEX on RPXR cell viability. B. Effects of TCS on RPXR cell viability. C. Effects of RIF on DPX-2 cell viability. D. Effects of TCS on DPX-2 cell viability.

Appendix 6:
Functional Hepatic Transport May Not Contribute Significantly to Triclosan-Induced Hypothyroxinemia in Rats: Description of Experiments and Results

Katie B. Paul[1], Kristina K. Wolf[2], Kim L. R. Brouwer[2], Michael J. DeVito[3], Kevin M. Crofton[4]

[1]Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [2]Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [3]National Toxicology Program, National Institute of Environmental Health Sciences, RTP, NC; [4]Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, RTP, NC, 27711

1. Abstract

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol;(TCS)] decreased serum thyroxine (T4) in rats and upregulated Phase I and II hepatic metabolism in rats in our previous work. The role of hepatic transporters in the mode of action for TCS-induced hypothyroxinemia has not been evaluated. This work tested the hypothesis that TCS decreases T4 via activation of the pregnane X and constitutive androstane receptors (PXR and CAR) resulting in up-regulation of thyroid hormone transport. Rat sandwich-cultured hepatocytes (SCH) were treated with TCS (0-30µM; non-cytotoxic as determined by lactate dehydrogenase activity) for 10 min or 48 hr prior to measuring uptake and biliary clearance (Cl_b) of estradiol-17β-glucuronide (E217G; Oatp/Mrp2 probe) and digoxin (Oatp1a4/Mdr1 probe) using B-CLEAR® technology. Minor (<20%) effects on E217G cell accumulation, but no significant differences in E217G Cl_b, were observed. TCS did not alter digoxin cell accumulation, but decreased digoxin Cl_b in a dose-dependent manner (48% at 30 µM TCS). Thus, TCS may modestly inhibit Mdr1-mediated biliary excretion. Protein content for Cyp3a1, Oatp1a1, Oatp1a4, Mdr1, Mrp2, Mrp3, and Mrp4 in SCH was assessed by immunoblotting. TCS increased Cyp3a1 at all doses; Oatp1a4 and Mrp2 protein were slightly increased. These data suggest that while TCS may interact with CAR and PXR to up-regulate hepatic catabolism and decrease circulating T4 concentrations *in vivo*, TCS has minimal effects on the expression and function of hepatic transporters associated with transport of thyroid hormone an an *in vitro* model.

2. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxyphenylether) (TCS) is a chlorinated phenolic bacteriostat found as an active ingredient in many personal care products, and as a surface water contaminant (ng/L) (Kolpin *et al.*, 2002). Initial work addressed the data gap: is TCS a

mammalian thyroid hormone disruptor? TCS treatment in a 4-day short-term assay produced dose-dependent decreases in thyroxine (T4) in weanling female Long Evans rats (Crofton *et al.*, 2007). In order to assess the potential hazard posed to human health by TCS, a mode-of-action hypothesis was developed using *in vivo* rat models. To test the hypothesized mode-of-action that TCS decreases T4 via activation of the pregnane X and constitutive androstane receptors (PXR, CAR) via subsequent up-regulation of hepatic catabolism and transport of thyroid hormone, weanling female Long-Evans rats received TCS po (0-1000 mg/kg/day) for 4 days. Pentoxyresorufin-O-deethylase (PROD) and uridine diphosphate glucuronyltransferase (UGT) enzyme activities were measured in liver microsomes. qRT-PCR was used to measure mRNA expression of cytochrome P450s *1a1*, *2b2*, and *3a*, UGTs *1a1*, 1a6, and *2b5*, sulfotransferases *1c1* and *1b1*, and hepatic transporters *Oatp1a1* and *1a4*, *Mrp2*, and *Mdr1b*. PROD activity increased ~800 percent and T4-glucuronidation increased 2-fold at 1000 mg/kg/day. *Cyp2b2*, *Cyp3a*, *Ugt1a1*, and *Sult1c1* mRNA expression levels were induced 2-fold, 4-fold, 2.2-fold, and 2.6-fold at 300 mg/kg/day, respectively. No changes were observed in mRNA levels for any hepatic transporters. The current data only support the hypothesis that TCS up-regulates Phase II hepatic catabolism of thyroid hormone to produce hypothyroxinemia *in vivo*. Though the mRNA expression results do not suggest that changes in transporter expression contribute to the observed T4 decreases, these results do not exclude the possibility that hepatic transporter protein expression and activity may be altered by TCS treatment via receptor mediated post-translational modifications (Johnson *et al.*, 2002b).

Increased *Cyp3a1/23* expression and *in vitro* receptor reporter assays (Jacobs *et al.*, 2005) suggest that TCS activates PXR, perhaps altering expression and/or activity of PXR-regulated *Oatp1a4* (Kretschmer *et al.*, 2005). It is important to measure protein activity of

transporters, as PXR ligands that induce Cyp3a may increase Mrp2 protein without increasing *Mrp2* expression (Johnson & Klaassen 2002). Due to the functional overlap between PXR and CAR (Faucette *et al.*, 2006), it is also possible that TCS activates CAR to increase expression of *Cyp3a1/23*. CAR modulates expression and function of Mrp2, Mrp3, and Mrp4 canalicular transporters (Cherrington *et al.*, 2002; Johnson *et al.*, 2002a; Johnson et al., 2002b; Klaassen *et al.*, 2005). TCS may interact with CAR and/or PXR, and up-regulated markers of hepatic catabolism *in vivo*, thus defining a major area of uncertainty in the proposed MOA for TCS-induced hypothyroxinemia: the potential contribution of up-regulated hepatic transport.

The objective of the current work was to determine the contribution of functional Phase III hepatic transport to TCS-induced hypothyroxinemia in rat hepatocytes to uncover potential effects of TCS on hepatic transporters that could not be evaluated with qRT-PCR alone. Sandwich-cultured rat hepatocytes (SCRH) were used to examine hepatic uptake activity of organic anion transporting polypeptides (Oatps) and efflux activity of multidrug resistance/resistance-associated proteins (Mdr1, Mrp2) following TCS exposure. Two hypotheses were tested: the protein expression and activity of the uptake transporters Oatp1a1 and 1a4 are increased by TCS treatment; and, protein expression and cannalicular transport function of the efflux transporters Mdr1 and Mrp2 are increased by TCS treatment. Uptake and biliary clearance were assessed in sandwich-cultured rat hepatocytes (SCRH) by assessing the accumulation of two substrates, estradiol-17β-glucuronide and digoxin, to probe the functional activity of Oatp1a1/1a4 and Mrp2, and Oatp1a4 and Mdr1, respectively (Liu *et al.*, 1999; Marion *et al.*, 2011; Swift *et al.*, 2010; Turncliff *et al.*, 2006; Wolf *et al.*, 2008).

3. Methods

A. Materials

Collagenase (Type 1, class 1) was obtained from Worthington Biochemicals (Lakewood, NJ). Dulbecco's modified Eagle's medium (DMEM, no phenol red), insulin, MEM non-essential amino acids solution (100x), L-glutamine, penicillin G-streptomycin solution were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum, sodium taurocholate, digoxin, Triton X-100, dexamethasone, methanol, Hanks' balanced salts solution (HBSS) modified with (H-1387) or without (H-4891) calcium chloride, bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). BioCoat™ collagen I plates, Matrigel™ basement membrane matrix, and ITS^{+}™ (insulin/transferrin/selenium) culture supplement were purchased from BD Biosciences Discovery Labware (Bedford, MA). [^{3}H]- estradiol-17β-glucuronide (40 Ci/mmol, >97% purity) and [^{3}H]-digoxin (40 Ci/mmol, >97% purity) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Bio-Safe II™ liquid scintillation cocktail was obtained from Research Products International (Mt. Prospect, IL). Bicinchoninic acid (BCA) protein assay reagents and BSA for the protein assay standard were purchased from Pierce Chemical Co. (Thermo Scientific, Rockford, IL).

B. Source of rat hepatocytes

Hepatocytes were obtained from male Wistar rats (200–325 g) from Charles River Laboratories, Inc. (Raleigh, NC). Animals colony rooms were maintained at 21 ± 2°C with 50 ± 10 % humidity on a photo-period of 12L:12D. Food and water were provided *ad libitum* prior to surgical extraction of cells. All animal procedures were compliant with the guidelines of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

C. Isolation and *In Vitro* Culture of Primary Rat Hepatocytes

Hepatocytes were isolated from male Wistar rats (215.8 – 287.4 g) by collagenase perfusion (LeCluyse *et al.*, 1996). Cell viability was determined by trypan blue exclusion to be ≥ 91.5%. Hepatocytes were seeded at a density of 1.75×10^6 cells per well in 6-well BioCoat™ plates in DMEM supplemented with 2 mM L-glutamine, 1% (v/v) MEM non-essential amino acids, 100 units penicillin G sodium/mL, 100 μg streptomycin sulfate/mL, 1 μM dexamethasone, 5% (v/v) fetal bovine serum, and 10 μM insulin (Day 0 in culture), and cultured in a humidified incubator (95% O_2, 5% CO_2) at 37°C. Hepatocytes were allowed a 2 hr incubation time for attachment before media was aspirated to remove unattached and nonviable cells and replaced with fresh media. Twenty-four hours after seeding (Day 1), hepatocytes were overlaid with Matrigel™ basement membrane matrix (0.25 mg/mL) in 2 mL/well cold, serum-free DMEM containing 2 mM L-glutamine, 1% (v/v) MEM non-essential amino acids, 100 units penicillin G sodium/mL, 100 μg streptomycin sulfate/mL, 0.1 μM dexamethasone, and 1% (v/v) ITS^{+}™ (note the absence of fetal bovine serum). The culture medium was changed every 24 hr until uptake experiments or protein collection were performed on Day 4 in culture.

D. Chemical Treatment

Most of the experiments utilized a 48 hr pretreatment period to allow enough time for a nuclear receptor-mediated up-regulation of hepatic transporter function. For dexamethasone (DEX) only, treatment began on Day 1 in culture and concluded on Day 3 in culture to allow for a 24 hr wash-out period prior to uptake experiments. Triclosan (TCS) and phenobarbital (PB) treatments were from Day 2 to Day 4. Starting on Day 2 in culture, SCRH were pretreated with TCS or PB via addition of these compounds to the media applied to replenish

the SCRH. TCS and DEX were dissolved in DMSO at stock concentrations of: 100 mM. Phenobarbital is a sodium salt and was prepared at a stock concentration of 100 mM in media. The chemical stock solutions were then used to generate volumes of warmed media at concentrations of 10-100 µM that were applied to the SCRH.

E. Cytotoxicity Experiments

To determine the cytotoxicity of TCS for different exposure durations, a time-course experiment was completed with TCS at 1, 10, and 30 µM. For the 72 hr timecourse, SCRH were treated with TCS in the media starting on Day 1 in culture, 4 hrs after being overlaid with Matrigel. At each subsequent 24 interval, i.e. Day 2, Day 3, and Day4, cytotoxicity was assessed by lactate dehydrogenase (LDH) activity assay (Roche Applied Science, Mannheim, Germany). A 2% Triton-X stock, diluted with media, was used as the cytotoxicity control (100% cell death), while media from untreated wells of SCRH was used as the negative control. For the 48 hr, 24 hr, and 2 hr timecourses, TCS treatment began on Day 2, Day 3, and Day 4. The LDH assay was run according to the directions of the manufacturer; briefly, 2 µL aliquots of media from treated SCRH wells were added to 100 µL of mixed LDH reagents in a 96 well plate. The plate was incubated for 25 min at room temperature in the dark before being read on a warmed spectrophotometric plate reader at 492 nm. Data were standardized to the Triton-X control per the following equation:

$$\left(\frac{Average\ Abs_{492} - Average\ Negative\ Control\ Abs_{492}}{Average\ Triton - X\ Abs_{492}}\right) * 100 = Percent\ cell\ death$$

F. Uptake Experiments

The method for determining the uptake and substrate accumulation of substrates in SCRH was performed as previously described (Liu *et al.*, 1999; Marion *et al.*, 2011; Turncliff *et al.*, 2004; Wolf *et al.*, 2008). All treatments were performed in technical

duplicates in three separate experiments. In summary, hepatocytes were washed twice with 2 mL warm Hanks' balanced salts solution (HBSS) that contained Ca^{2+} or Ca^{2+}-free HBSS, and subsequently incubated with 2 mL of the same buffer for 10 min at 37°C. Incubation of the SCRH with Ca^{2+}-free HBSS results in a loss of the tight junctions necessary to maintain bile canalicular networks enabling examination of the accumulation of substrate in cells alone. Conversely incubation with the Ca^{2+}-containing HBSS maintained tight junctions and biliary networks, allowing for assessment of substrate accumulation in cells and bile. The buffer was removed, and the cells were incubated for 10 min at 37°C with 1.5 mL of [^{3}H]-Estradiol-17β-glucuronide (E217G) (1 μM) or [^{3}H]-digoxin (10 μM) Ca^{2+}-containing HBSS. The SCRH were rinsed vigorously three times with 2 mL ice-cold Ca^{2+}-containing HBSS after incubation with the tritiated substrate. For rinsing of [^{3}H]-digoxin-treated SCRH, 10 μM non-radiolabeled digoxin was added to the rinsing buffer to reduce nonspecific binding as described previously (Annaert *et al.*, 2001). E217G- and digoxin-treated hepatocytes were lysed with 1 mL 0.5% (v/v) Triton X-100 in phosphate-buffered saline by placing plates on an orbital shaker for a minimum of 20 min at room temperature. The samples were analyzed by liquid scintillation spectroscopy in a Packard Tri-Carb scintillation counter (PerkinElmer Life and Analytical Sciences). A sample was also reserved and kept at -20ºC for protein determination.

G. Total Protein Measurements

The uptake experiment data were normalized to the protein concentration in each well (lysed with Triton X-100) and determined in duplicate aliquots using BCA protein assay reagents as instructed by the manufacturer (Pierce Chemical). BSA was used as a standard (0.2 – 1 mg/mL).

H. Imunoblotting for protein content

Parallel experiments were run to collect total protein from SCRH treated with TCS, DEX, and PB. Instead of performing uptake experiments on Day 4, SCRH were incubated with 400 µL lysis buffer (Complete Protease Inhibitor™ (Roche Diagnostics), 1 mM EDTA, 1% SDS) and scraped off of the plates. These samples were then briefly sonicated and centrifuged at 900 rpm for 5 min at room temperature. Total protein measurements were performed on these lysates. Samples were prepared for gel electrophoresis such that a 30 µL sample applied to each well contained 50 µg of protein, 10% 0.5 M DTT, 7.5 uL of Laemmli Sample Buffer, and Lysis Buffer. Samples were stored at -20ºC until use.

Target proteins were resolved by electrophoresis with NuPAGE 4-12% Bis-Tris Gels (Invitrogen; Carlsbad, CA), followed by transfer to PVDF membrane prior to immunoblotting. The following antibodies were used for immunoblotting: Oatp1a1 and Oatp1a4 (Chemicon/Millipore); Mdr1 (Santa Cruz Biotechnology, Inc.); Mrp3 was a generous gift to the Brouwer laboratory from Dr. Yuichi Sugiyama; Mrp4 and Mrp2 (Alexis Biochemicals); Cyp3a1 (Xenotech, Inc.); Beta-actin for use as a loading control (Sigma-Aldrich). Immunoblot detection was performed with chemiluminescence reagents (SuperSignal/West Dura; Pierce Chemical, Rockford, IL).

I. Data Analysis

The BEI (%) and unbound intrinsic biliary clearance (intrinsic $Cl_{biliary}$, mL/min/kg) were calculated using B-CLEAR® technology (Qualyst, Inc., Raleigh, NC; (Liu et al., 1999):

$$BEI = \left(\frac{(Accumulation_{Cells+Bile} - Accumulation_{Cells})}{Accumulation_{Cells+Bile}}\right) * 100$$

where substrate accumulation in the cell plus bile compartments was determined in hepatocytes preincubated in buffer containing Ca^{2+}, and the accumulation of substrate in cells alone was determined in hepatocytes preincubated with Ca^{2+}-free buffer. Biliary clearance was given by:

$$Cl_{biliary} = \frac{Accumulation_{Cells+Bile} - Accumulation_{Cells}}{AUC_{media}}$$

where AUC_{media} represents the area under the substrate concentration-time curve, determined by multiplying the incubation time (10 min) by the concentration of substrate used (1 µM). Intrinsic $Cl_{biliary}$ values were converted to mL/min/kg based on 200 mg protein/g of liver and 40 g liver/kg of rat body weight (Seglen, 1976).

For uptake experiments, main effects of chemical treatment were determined by one-way ANOVAs (SAS 9.1, SAS Institute, Cary, NC), followed by a mean contrast testing with Duncan's New Multiple Range Test with a significance level of $p<0.05$ with chemical concentration as the independent variable.

4. Results and Discussion

A. LDH assay and TCS cytotoxicity

LDH activity assays were conducted to determine the cytotoxicity of TCS and the non-cytotoxic concentrations that could be used for uptake studies in the SCRH. The greatest cytotoxicity was observed with 100 µM TCS, especially when SCRH were exposed starting on Day 1 of culture (Figure A6.1). The exposure paradigm employed in uptake experiments, i.e. exposure for 48 hours beginning on Day 2 in culture, demonstrated that 100 µM TCS would have resulted in about 40% cell death. Only 0-30 µM TCS were used in subsequent experiments.

B. Immunoblotting for protein content

Preliminary results from two sets of Western blots suggest that TCS did not greatly affect the protein expression of hepatic transporters. A representative set of blots is shown in Figure A6.2, with densitometry average values presented in Table A6.1. Densitometry identified small (20%) increases in Mrp2 expression and 10-50% increases in Oatp1a4 expression, though these changes did not appear to be dose-dependent. Treatment with DEX produced the most striking, dose-dependent changes; for 10 and 100 µM, DEX increased Mrp2 to 158 and 223 percent of control, Mdr1 to 127 and 141 percent of control, and for 100 µM only DEX increased Mdr1 to 124 percent of control. Like TCS, PB at the doses used had only limited, if any, effects on the expression of hepatic transporters. For PB at 30 and 100 µM, PB increased Mrp2 expression by about 20 percent. It seems likely that changes of 20% or less in protein content, detected by densitometry, may not be reflective of significant changes.

Cyp3a1 protein content was also assessed to demonstrate that TCS entered the cells and behaved similarly to our *ex vivo* observations, and to demonstrate that DEX and PB functioned correctly in the SCRH. DEX induced a dose-dependent increase in protein content; at 10 and 100 µM, DEX increased Cyp3a1 content to 252 and 300 percent of control. PB also increased Cyp3a1, though less dramatically, to 154 and 168 percent of control. TCS also had a moderate effect on Cyp3a1 protein expression; though not dose-dependent, TCS increased Cyp3a1 content by about 50-60% for all of the doses tested. This was a key finding because it demonstrated that TCS entered the cells, induced Cyp3a1 indicative of PXR and/or CAR activation, and behaved similarly *in vitro* and *in vivo*, as *Cyp3a1* mRNA expression was increased *in vivo*.

C. Uptake experiments with E217G

Substrate accumulation for E217G was evaluated due to the specificity of E217G for Oatp uptake transporters and Mrp2, a canalicular transporter. This tested the hypothesis that pretreatment with TCS increased the functional activity of the Oatps and/or Mrp2, resulting in an increase in uptake and/or biliary excretion as measured by E217G disposition. The Oatp uptake transporters and Mrp2 are known to be involved in thyroid hormone uptake (Friesema *et al.*, 2005; Jansen *et al.*, 2005) and thyroid hormone conjugate excretion (Lecureux *et al.*, 2009; Wong *et al.*, 2005), respectively.

The cellular disposition of E217G was observed following 10 min of TCS pretreatment and 48 hr min of TCS pretreatment (Figure A6.3). Although significant effects of treatment were observed for both pretreatment paradigms, these effects were minimal and most likely not biologically significant. After 10 min of 1 µM TCS pretreatment, E217G accumulation in cells increased approximately 20%, associated with a decrease in BEI and $Cl_{b.}$ However, this effect did not appear to be dose-dependent, as accumulation in either the cells or cells+bile compartment was not increased 30 µM TCS. At 30 µM TCS, there was actually a decrease in the accumulation in cells+bile. The consistent trend with increasing TCS concentration was a decrease in BEI and Cl_b, suggesting that TCS treatment modestly decreased biliary elimination of E217G. A similar pattern was observed following 48 hr of TCS pretreatment, suggesting that incubation time did not impact the results. In fact, analysis using a two-way ANOVA with pretreatment time and dose as the independent variables demonstrated no effect of time on the results. The observation that TCS slightly decreased E217G disposition in this model is counter to the original hypothesis that TCS would interact with nuclear receptors to transcriptionally and/or post-translationally up-regulate transport function; but, the observed effects are extremely mild. Further, the

observation of decreased elimination of E217G may be obfuscated by competition with TCS-glucuronide. TCS is efficiently glucuronidated *in vivo* by rats, so TCS-G may compete with the labeled E217G substrate for disposition by Mrp2. Another confounding variable may be the off-target affinity of E217G for Mrp3, which would result in disposition of E217G into the media compartment, which was not tested.

Due to the minor effects observed with TCS, both that the effects were minor and that the effects suggested an impairment of clearance rather than an increase, prototypical receptor activators known to increase hepatic transporter expression were tested in the same paradigm. DEX and PB 48 hr pretreatments were employed to observe the potential effects on E217G disposition. Previously, DEX has been shown to up-regulate the protein content for Oatp1a4 and Mrp2 in sandwich-cultured hepatocytes (Turncliff et al., 2004), and PB has been shown to up-regulate Mrp3 and competitively inhibit Mrp2 function by its glucuronide metabolites (Chandra *et al.*, 2005). For PB at 30 and 100 µM, E217G accumulation was decreased in both cells + bile and cells compartments. Similarly for DEX at 10 and 100 µM, accumulation of E217G was decreased in both compartments in a dose-dependent manner. However, there were no apparent effects on BEI or Cl_b (data not shown). Though perplexing, this data suggests that increased Mrp3 function may be reflected in an apparent decrease in uptake, as substrate was likely excreted into the media. TCS did not appear to function like DEX and PB in culture. A mechanistic explanation is largely speculative. DEX is a model agonist for rat PXR, and PB is a model activator for rat CAR. Previous work suggests that TCS interacts with one or more of these receptors to up-regulate Phase I and II metabolism. However, TCS may not be as potent or efficacious of an activator of CAR and/or PXR.

D. Uptake experiments with digoxin

Substrate accumulation of digoxin was evaluated because it is a probe substrate for Oatp1a4 and Mdr1. As with the uptake experiments for E217G, digoxin disposition was assessed following pretreatment with TCS for 48 hr (Figure A6.5) to allow for potential nuclear receptor-mediated transcriptional and/or post-translational up-regulation of hepatic transport proteins. Again, the effects were not striking. There was a significant decrease of about 50% in the accumulation in the bile compartment for 30 µM TCS (computed as the difference between the cells + bile and cells compartments and not measured directly). A significant 48% decrease in Cl_b was noted for 30 µM TCS, suggesting that TCS slightly inhibited Mdr1-mediated biliary excretion. A decrease in biliary excretion activity would not support the hypothesis that increased hepatic transport activity led to increased rates of thyroid hormone elimination *in vivo*.

5. Conclusions

The data presented suggest that changes in hepatic transport do not significantly contribute to the observed TCS-induced hypothyroxinemia observed *in vivo*. Though exceedingly small increases in the cellular accumulation of E217G appeared statistically significant, it seems unlikely that these correspond to important biological changes in the context of chemicals that have inhibited hepatobiliary transport, such as bosentan and troglitazone, by much greater magnitudes (Lee *et al.*, 2010a; Lee *et al.*, 2010b; Marion *et al.*, 2007; Wolf *et al.*, 2010). The 50% decreased observed in Cl_b for digoxin following TCS exposure is discordant with the hypothesis that TCS could increase biliary elimination of substrates like thyroid hormones. However, there were several limitations to interpreting these observations. First, this assay is typically successfully employed to detect inhibitors of hepatic transport that might cause cholestatic injury *in vivo* (Swift et al., 2010). For TCS, we

were trying to observe the opposite effect: an induction of hepatic transport and biliary elimination that would suggest increased biliary elimination of thyroid hormones and thyroid hormone conjugates in vivo. Thus, we had limited experience to draw upon in trying to optimize the assay for our reverse purpose. The data from positive controls known to be prototypical CAR and PXR agonists that increase hepatic catabolism and hepatic transport was difficult to interpret. DEX and PB both seemed to significantly decrease the uptake and elimination of the E217G substrate. DEX is known to upregulate Oatp1a4 and Mrp2 protein expression (Turncliff et al., 2004), so this result was counter to our expectation that both uptake and elimination would increase. A potential hypothesis may be that both DEX and PB also increased the expression of Mrp3, which has some affinity for the E217G substrate, and these inducers may have been causing increased rates of substrate displacement back into the buffer, which was not tested for substrate concentrations. Perhaps collection and analysis of the buffer from uptake experiments would provide important information about how these prototypical receptor activators control hepatic transport. A final limitation may have been the choice of substrates. It may have been more instructive to have used ^{125}I-labeled thyroxine, as this is the compound of interest in the *in vivo* system. Then the relative displacement of labeled thyroxine could have been traced through the system following TCS pretreatment. If greater effects had been observed in protein content with Western blots, this may have been an interesting follow-up study with TCS. A more potent receptor agonist and known thyroid hormone disruptor, such as PCN, might produce a more pronounced result. Further investigation of how nuclear receptor agonists function in this model might be an interesting future experiment to understand how compounds that activate nuclear receptors might change the functional activity of hepatic transporters.

This work demonstrates a lack of effect of TCS on hepatic transport protein content and function. The only statistically significant effects suggest that TCS decreased biliary clearance of probe substrates, which is the opposite direction of activity expected if TCS were to up-regulated hepatic transport function *in vivo* to increase biliary elimination of thyroid hormones. Therefore, there is no evidence to suggest that modulation of hepatic transport function significantly contributes to the proposed mode-of-action for TCS-induced hypothyroxinemia *in vivo*. The key findings of this work were two-fold: TCS did not appear to affect hepatic transport, and TCS increased Cyp3a1 protein content in an *in vitro* model of rat liver. Despite a lack of effects on hepatic transport, TCS increased a biomarker associated with nuclear receptor activation in this *in vitro* model and also *in vivo*, demonstrating concordance across models.

6. Acknowledgements

This work was largely funded by a generous award from the Society of Toxicology Colgate-Palmolive Award for Student Research Training in Alternative Methods to K.B. Paul. Dr. Brouwer was a kind and patient laboratory host and mentor, who not only provided ideas and education about her methods, but also supported this work in her laboratory (NIH GM41935). Invaluable instruction and support from Drs. Kristina Wolf and Tracy Marion on sandwich-culture and experimentation made this work possible. Dr. Yiwei Rong performed the collagenase perfusions to obtain the hepatocytes used. K.B. Paul was supported by NIEHS T32-ES07126 and EPA CR833237 during completion of this work.

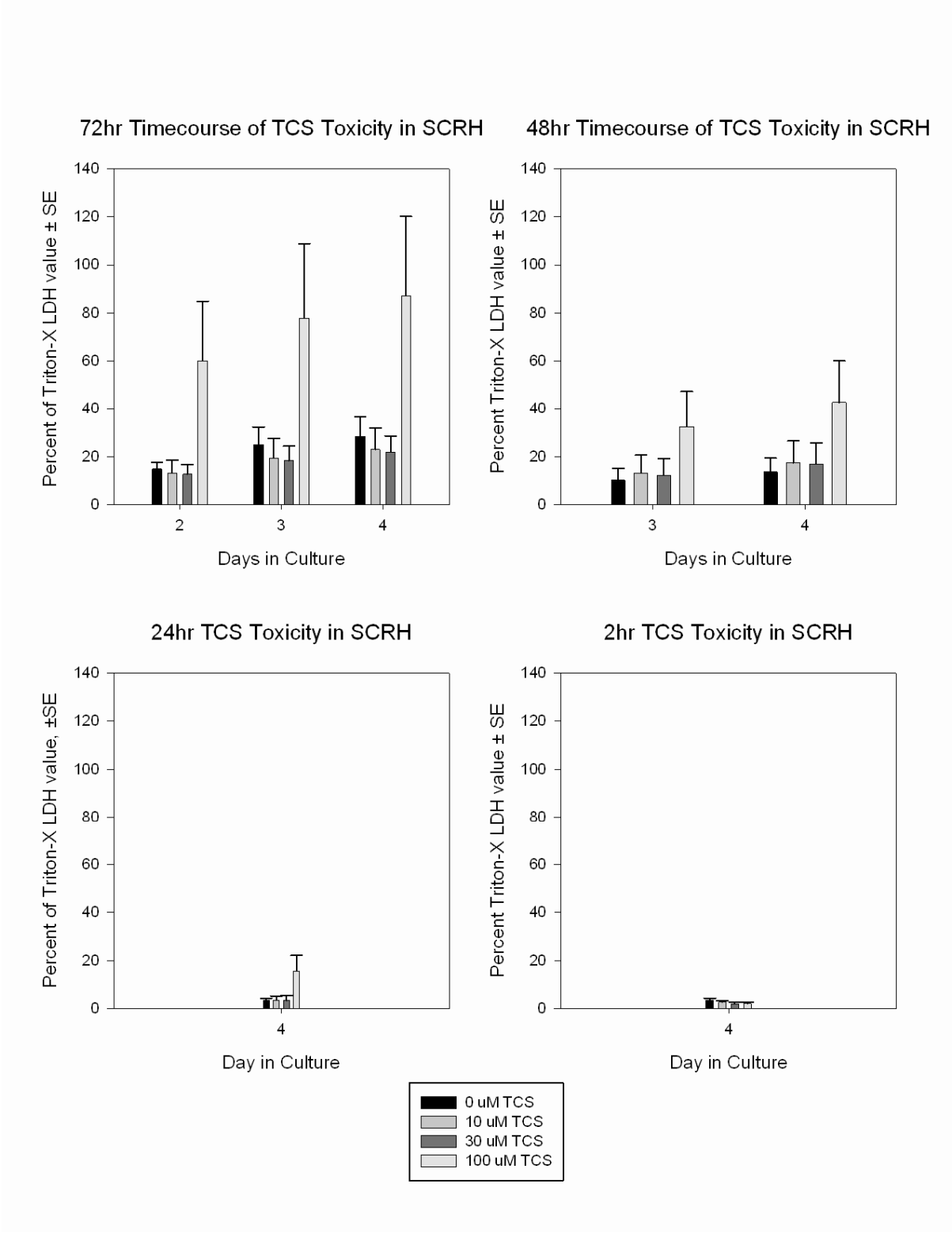


Figure A6.1. LDH activity assay data for TCS at 72, 48, 24, and 2 hr exposures.

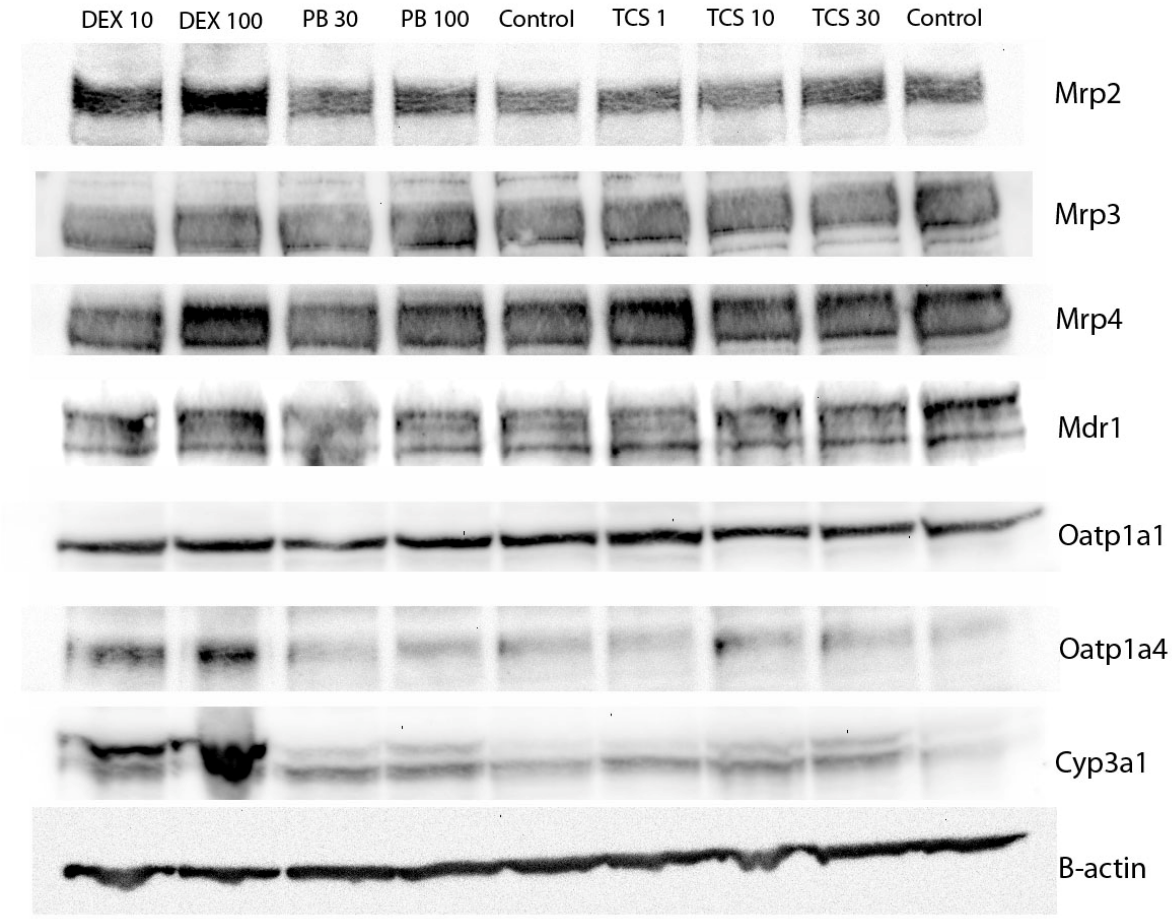


Figure A6.2. Results of immunoblotting for hepatic transporters and Cyp3a1.

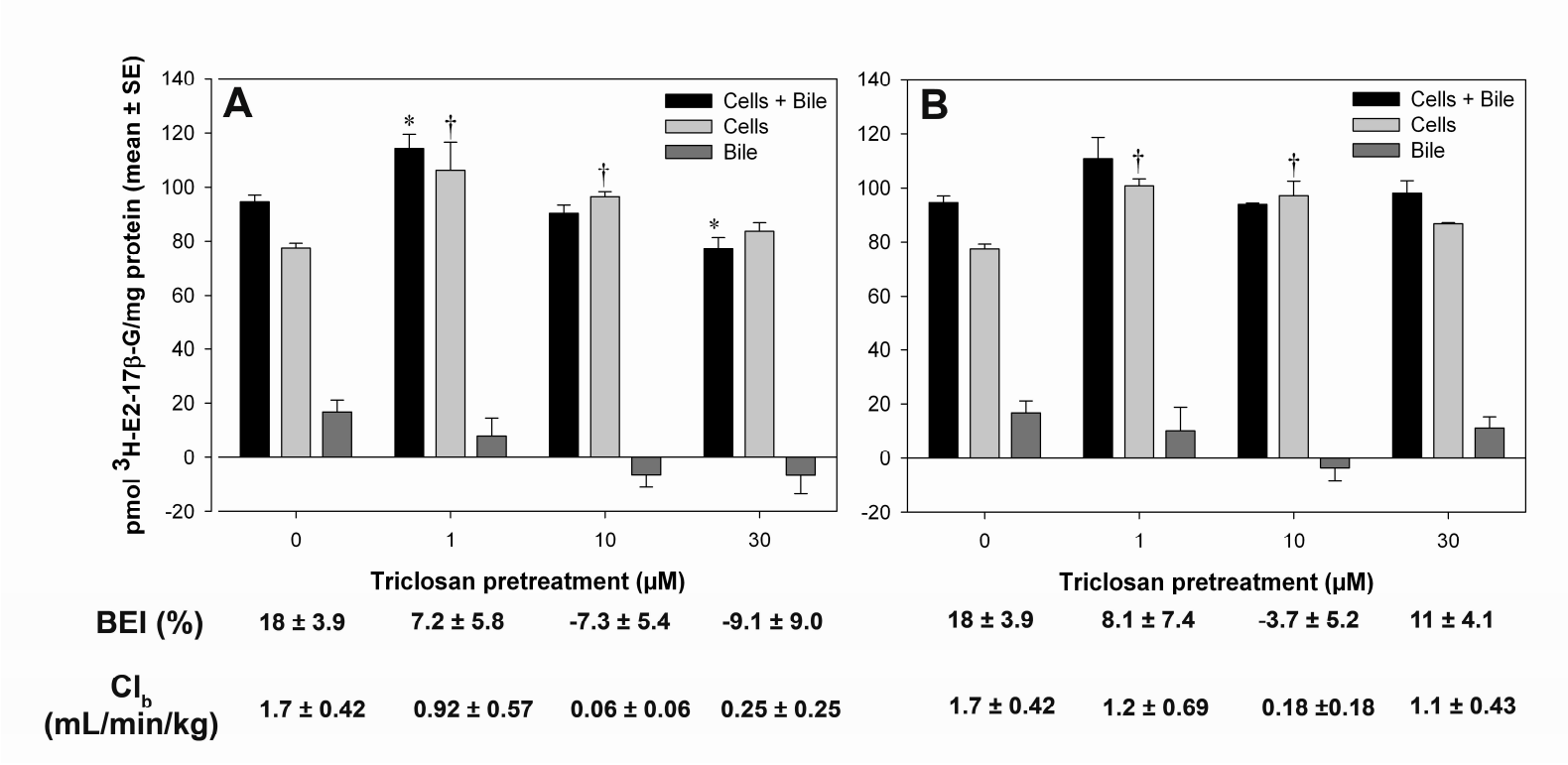


Figure A6.3. Disposition of E217G following A) 10 min pretreatment with TCS and B) 48 hr pretreatment with TCS. † = different from control value for cells only compartment; * = different from control value for cells + bile compartment ($p<0.05$).

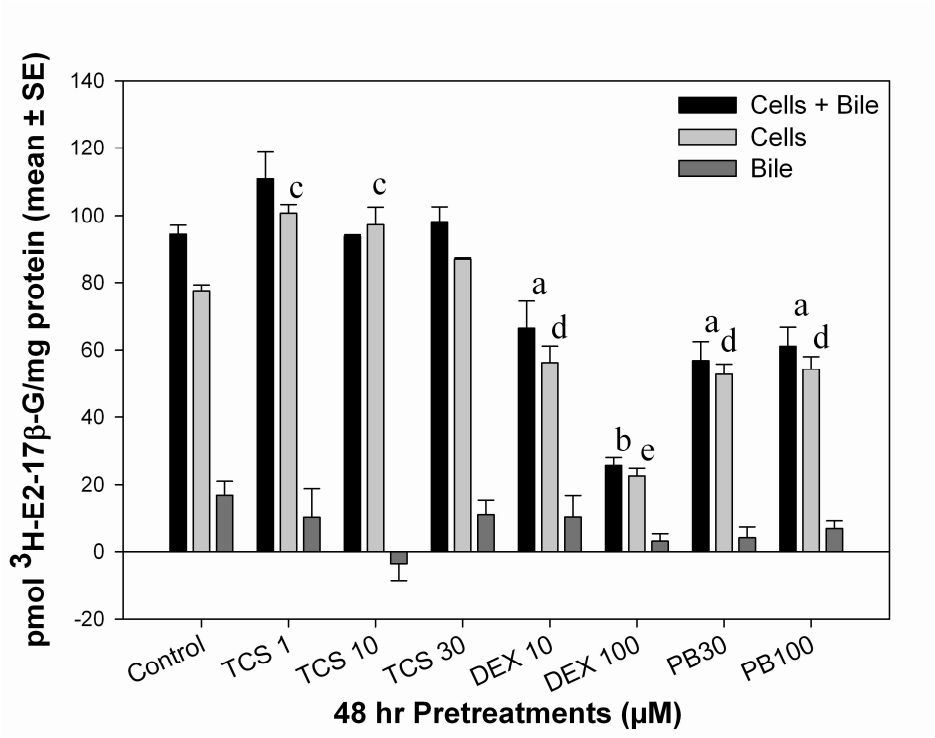


Figure A6.4. Disposition of E217G following 48 hr pretreatments with TCS (1, 10, and 30 μM), DEX (10 and 100 μM), and PB (30 and 100 μM). a = Significantly decreased from control value for cells + bile compartment; b = greater significant decrease from control value for cells + bile compartment; c = significantly increased from control value for cells + bile compartment.

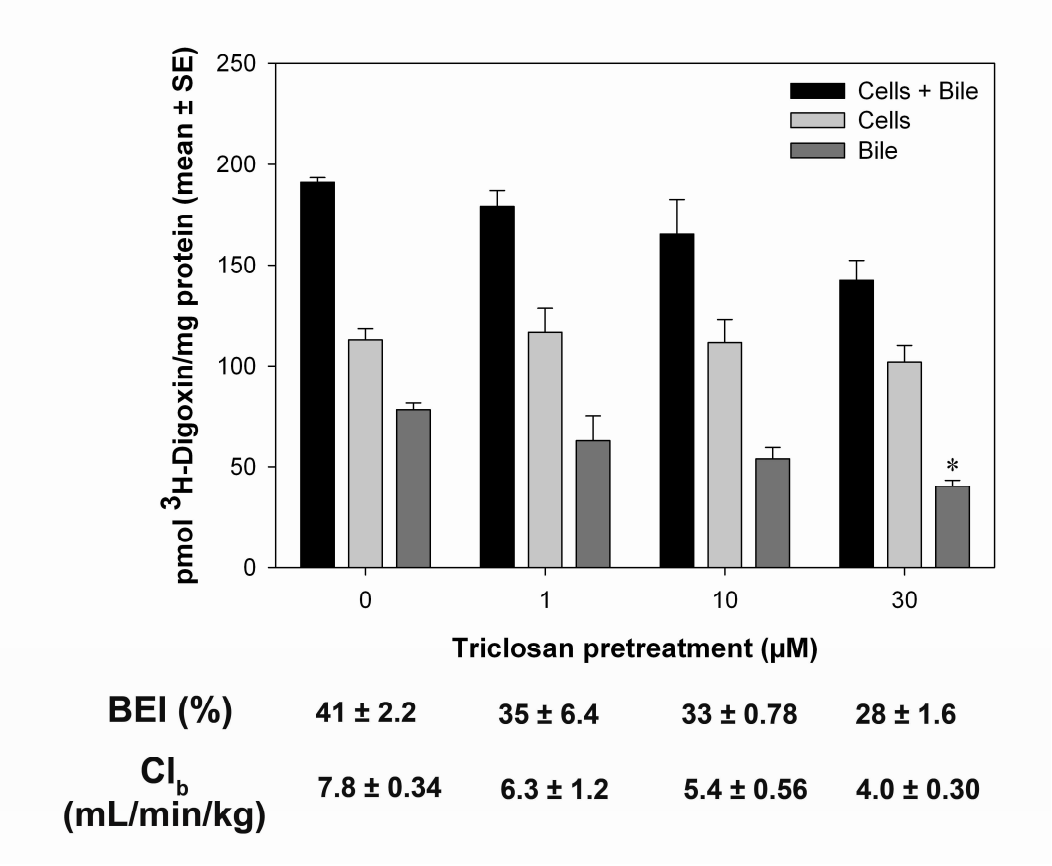


Figure A6.5. Disposition of digoxin following 48 hr of TCS pretreatment. * = significantly different from control value for bile compartment ($p<0.05$).

Relative Western Blot Densitometry
(percent of untreated control)

Chemical	µM	Mrp2	Mrp3	Mrp4	Mdr1	Oatp1	Oatp2	Cyp3a1
DEX	10	158	77	83	101	92	127	252
DEX	100	223	73	97	124	81	141	300
PB	30	117	86	105	81	89	102	154
PB	100	123	81	109	84	108	103	168
Control 1	0	90	82	100	92	117	119	119
TCS	1	97	90	92	91	134	109	164
TCS	10	107	85	87	101	98	154	147
TCS	30	119	86	92	84	89	119	150
Control 2	0	110	118	100	108	83	81	81
Average Control	0	100	100	100	100	100	100	100

Table A6.1. Relative densitometry for immunoblots (shown in Figure A6.1).

References

Annaert, P. P., R. Z. Turncliff, C. L. Booth, D. R. Thakker and K. L. Brouwer (2001). P-glycoprotein-mediated in vitro biliary excretion in sandwich-cultured rat hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **29**, 1277-1283.

Chandra, P., B. M. Johnson, P. Zhang, G. M. Pollack and K. L. Brouwer (2005). Modulation of hepatic canalicular or basolateral transport proteins alters hepatobiliary disposition of a model organic anion in the isolated perfused rat liver. *Drug metabolism and disposition: the biological fate of chemicals* **33**, 1238-1243.

Cherrington, N. J., D. P. Hartley, N. Li, D. R. Johnson and C. D. Klaassen (2002). Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mrp1, 2, and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. *The Journal of pharmacology and experimental therapeutics* **300**, 97-104.

Crofton, K. M., K. B. Paul, Hedge J.M. and D. M.J. (2007). Short-Term in Vivo Exposure to the Water Contaminant Triclosan: Evidence for Disruption of Thyroxine *Environmental Toxicology and Pharmacology* **24**, 194-197.

Faucette, S. R., T. Sueyoshi, C. M. Smith, M. Negishi, E. L. Lecluyse and H. Wang (2006). Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *The Journal of pharmacology and experimental therapeutics* **317**, 1200-1209.

Friesema, E. C., J. Jansen, C. Milici and T. J. Visser (2005). Thyroid hormone transporters. *Vitamins and hormones* **70**, 137-167.

Jacobs, M. N., G. T. Nolan and S. R. Hood (2005). Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). *Toxicology and applied pharmacology* **209**, 123-133.

Jansen, J., E. C. Friesema, C. Milici and T. J. Visser (2005). Thyroid hormone transporters in health and disease. *Thyroid* **15**, 757-768.

Johnson, D. R., S. S. Habeebu and C. D. Klaassen (2002a). Increase in bile flow and biliary excretion of glutathione-derived sulfhydryls in rats by drug-metabolizing enzyme inducers is mediated by multidrug resistance protein 2. *Toxicol Sci* **66**, 16-26.

Johnson, D. R. and C. D. Klaassen (2002b). Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. *Toxicol Sci* **67**, 182-189.

Klaassen, C. D. and A. L. Slitt (2005). Regulation of hepatic transporters by xenobiotic receptors. *Current drug metabolism* **6**, 309-328.

Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber and H. T. Buxton (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environmental science & technology* **36**, 1202-1211.

Kretschmer, X. C. and W. S. Baldwin (2005). CAR and PXR: xenosensors of endocrine disrupters? *Chemico-biological interactions* **155**, 111-128.

LeCluyse, E. L., P. L. Bullock, A. Parkinson and J. H. Hochman (1996). Cultured rat hepatocytes. *Pharm Biotechnol* **8**, 121-159.

Lecureux, L., M. Z. Dieter, D. M. Nelson, L. Watson, H. Wong, B. Gemzik, C. D. Klaassen and L. D. Lehman-McKeeman (2009). Hepatobiliary disposition of thyroid hormone in Mrp2-deficient TR- rats: reduced biliary excretion of thyroxine glucuronide does not prevent xenobiotic-induced hypothyroidism. *Toxicol Sci* **108**, 482-491.

Lee, J. K., T. L. Marion, K. Abe, C. Lim, G. M. Pollock and K. L. Brouwer (2010a). Hepatobiliary disposition of troglitazone and metabolites in rat and human sandwich-cultured hepatocytes: use of Monte Carlo simulations to assess the impact of changes in biliary excretion on troglitazone sulfate accumulation. *The Journal of pharmacology and experimental therapeutics* **332**, 26-34.

Lee, J. K., M. F. Paine and K. L. Brouwer (2010b). Sulindac and its metabolites inhibit multiple transport proteins in rat and human hepatocytes. *The Journal of pharmacology and experimental therapeutics* **334**, 410-418.

Liu, X., E. L. LeCluyse, K. R. Brouwer, L. S. Gan, J. J. Lemasters, B. Stieger, P. J. Meier and K. L. Brouwer (1999). Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* **277**, G12-21.

Marion, T. L., E. M. Leslie and K. L. Brouwer (2007). Use of sandwich-cultured hepatocytes to evaluate impaired bile acid transport as a mechanism of drug-induced hepatotoxicity. *Mol Pharm* **4**, 911-918.

Marion, T. L., C. H. Perry, R. L. St Claire, W. Yue and K. L. Brouwer (2011). Differential Disposition of Chenodeoxycholic Acid versus Taurocholic Acid in Response to Acute Troglitazone Exposure in Rat Hepatocytes. *Toxicol Sci*.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol* **13**, 29-83.

Swift, B., N. D. Pfeifer and K. L. Brouwer (2010). Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug Metab Rev* **42**, 446-471.

Turncliff, R. Z., P. J. Meier and K. L. Brouwer (2004). Effect of dexamethasone treatment on the expression and function of transport proteins in sandwich-cultured rat hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 834-839.

Turncliff, R. Z., X. Tian and K. L. Brouwer (2006). Effect of culture conditions on the expression and function of Bsep, Mrp2, and Mdr1a/b in sandwich-cultured rat hepatocytes. *Biochem Pharmacol* **71**, 1520-1529.

Wolf, K. K., K. R. Brouwer, G. M. Pollack and K. L. Brouwer (2008). Effect of albumin on the biliary clearance of compounds in sandwich-cultured rat hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **36**, 2086-2092.

Wolf, K. K., S. Vora, L. O. Webster, G. T. Generaux, J. W. Polli and K. L. Brouwer (2010). Use of cassette dosing in sandwich-cultured rat and human hepatocytes to identify drugs that inhibit bile acid transport. *Toxicol In Vitro* **24**, 297-309.

Wong, H., L. D. Lehman-McKeeman, M. F. Grubb, S. J. Grossman, V. M. Bhaskaran, E. G. Solon, H. S. Shen, R. J. Gerson, B. D. Car, B. Zhao and B. Gemzik (2005). Increased hepatobiliary clearance of unconjugated thyroxine determines DMP 904-induced alterations in thyroid hormone homeostasis in rats. *Toxicol Sci* **84**, 232-242.

Appendix 7:
Reconstituted Freeze-Dried Wastewater Effluents Activate the Human Pregnane-X Receptor in Receptor Reporter Assays in DPX-2 cells

Initial consideration of three different freeze-dried effluent samples (Marc Mills, Shoji Nakayama, U.S. EPA) demonstrated remarkable hPXR activation activity. Freeze-dried effluents were collected at wastewater treatment plant outflow at two different wastewater treatment plants (Cincinnati, OH), prior to dilution of the effluent into surface water. The samples were then freeze-dried, and this sample was solubilized in media for use in receptor-reporter assays. The original constitution of this effluent is denoted as "1X," with all other concentrations based on this benchmark. Examination of these three effluents was performed in duplicate on three separate occasions (n=3). The hPXR activation in the receptor-reporter assay by 1X effluent is 3- to 5-fold the vehicle control. This represents a significant avenue for continued research, including further characterization of environmental samples with *in vitro* receptor reporter assays, but also in characterization of what environmental contaminants might be contributing PXR activity, like TCS and other known pharmacological agents that are hPXR agonists.

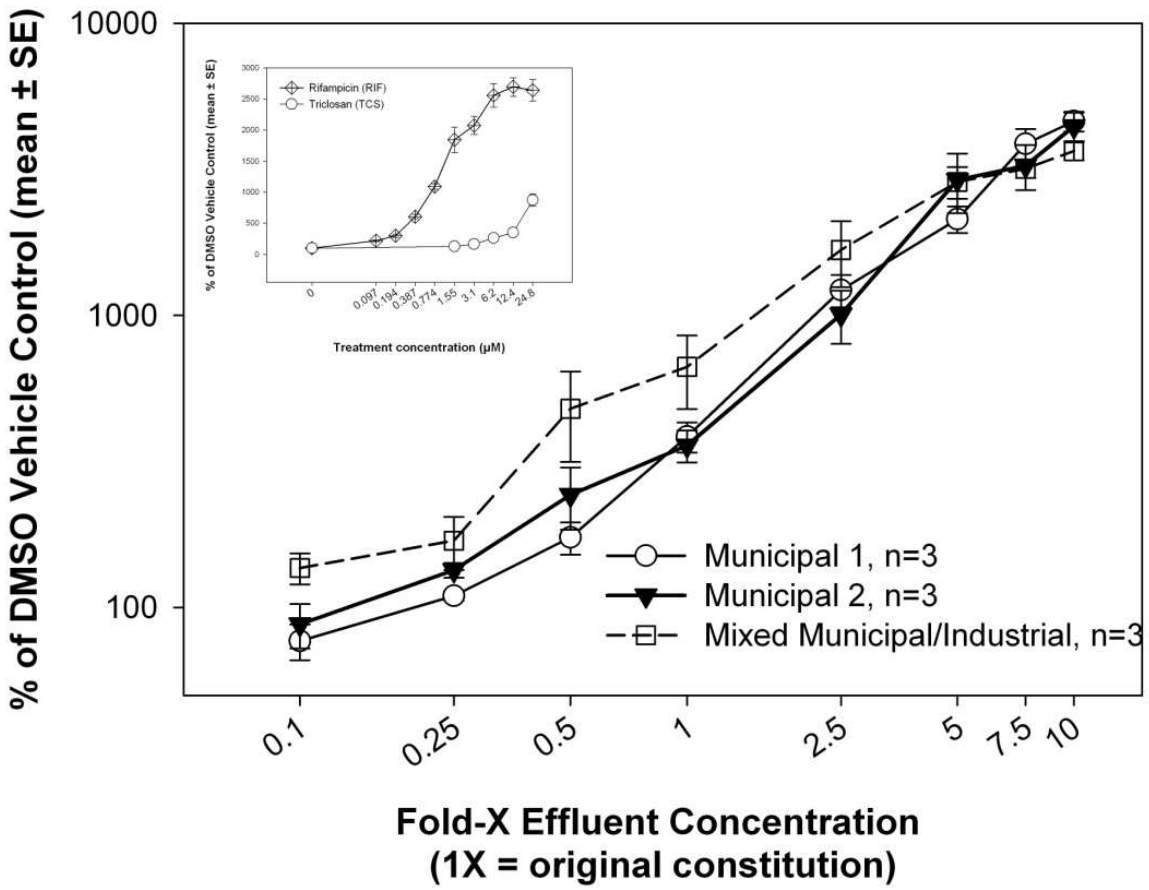


Figure A7.1. Three freeze-dried wastewater treatment plant effluent samples activated hPXR.