INTERACTIONS AMONG ENDOCRINE-DISRUPTING CHEMICALS

CONCENTRATIONS OF BISPHENOL A AND ESTRADIOL ARE ELEVATED BY EXPOSURE TO ENDOCRINE-DISRUPTING CHEMICALS

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TITLE: Concentrations of bisphenol A and estradiol are elevated by exposure to endocrine-disrupting chemicals

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Abstract

Endocrine-disrupting chemicals (EDCs) are synthetic substances that perturb estrogen, androgen, and thyroid systems, thereby impacting development, reproduction, behaviour, and general health. People are concurrently exposed to numerous EDCs from diverse sources, including consumer products, personal care products, medical devices, and environmental media. I explored impacts of [1] triclosan (a synthetic biocide), [2] butyl paraben and [3] propyl paraben (antimicrobial preservatives), and [4] tetrabromobisphenol A (a flame retardant) on bisphenol A (BPA) and 17β-estradiol (E2) concentrations *in vivo*. I developed a novel protocol that measured acute effects of these four EDCs upon radiolabeled ^{14}C-BPA or ^{3}H-E2 in blood serum and tissues, and natural E2 in urine. First I showed that BPA, the monomer of polycarbonate plastics, localizes to the reproductive organs of female mice following low-dose dietary exposure. Subsequently, I demonstrated that exposure to any one of the four EDCs elevated ^{14}C-BPA concentrations in blood serum, reproductive organs, and other peripheral tissues of female and male mice. Such exposure also elevated concentrations of natural E2 in urine. Finally, I showed that concurrent exposure to all four EDCs, in addition to diethylhexyl phthalate (a plasticizer), can elevate concentrations of ^{14}C-BPA and E2 at much lower doses than required when given alone. These data show that endocrine-disrupting chemicals interact *in vivo*, and that they can modulate the toxicokinetics of BPA and disrupt natural estrogen homeostasis. This is consistent with evidence that EDCs compete for access to enzymes that are critical for their metabolism and estrogen regulation, such as cytochrome p450, sulfotransferase, UDP-glucuronosyltransferase, and hydroxysteroid

dehydrogenase. Given the detrimental reproductive and carcinogenic effects of persistently elevated estrogen activity, the findings of these studies emphasize the importance of further investigating adverse health outcomes of chemical mixtures. Furthermore, studies of multiple chemicals should be considered when assessing risk and determining regulatory exposure limits.

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List of Abbreviations

Chemicals and Substances

17β-estradiol	E2, E_2
17β-estradiol glucuronide	E2G
17β-estradiol sulfate	E2S
β-glucuronidase	β-gluc
Bisphenol A	BPA
Butyl paraben	BP
Di(2-ethylhexyl) phthalate	DEHP
Estriol	E3
Estrone	E1
Estrone sulfate	E1S
Ethyl paraben	EP
Horseradish peroxidase	HRP
Methyl paraben	MP
Mono(2-ethyl-5-carboxypentyl) phthalate	MECPP
Mono(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP
Mono(2-ethyl-5-oxohexyl) phthalate	MEOHP
Mono(2-ethylhexyl) phthalate	MEHP
N-Methyl-N-trimethylsilyl-trifluoroacetamide	MSTFA
Peanut butter	PB

Phosphate-buffered saline	PBS
Progesterone	P4
Propyl paraben	PP
Radiolabelled bisphenol A	^{14}C-BPA
Tetrabromobisphenol A	TBBPA
Triclosan	TCS
Tritium-labeled 17β-estradiol	^{3}H-E_2

Receptors and Enzymes

Catechol-*O*-methyltransferase	COMT
Cytochrome P450	CYP
Estrogen receptor	ER
Estrogen-related receptor gamma	ERRγ
G protein-coupled estrogen receptor 1	GPER
G protein-coupled receptor 30	GPR30
Hydroxysteroid dehydrogenase	HSD
Membrane estrogen receptor alpha	mERα
Steroid sulfatase	STS
Sulfotransferase	SULT
UDP-Glucuronosyltransferase	UGT

Toxicological Terms and Programs

Chemical Abstracts Service	CAS
Collaborative Estrogen Receptor Activity Prediction Project	CERAPP
Endocrine Disruptor Screening Program	EDSP
Endocrine-disrupting chemical	EDC
Environmental Protection Agency	EPA
Lowest observed adverse effect level	LOAEL
National Health and Nutrition Examination Survey	NHANES
National Toxicology Program	NTP
Tolerable daily intake	TDI

Methods and Statistics

Analysis of variance	ANOVA
Enzyme-linked immunosorbent assay	ELISA
Gas chromatography-mass spectrometry	GC-MS
Intraperitoneal	ip
Intravenous	i.v.
Mass selective detector	MSD
Michigan Cancer Foundation-7	MCF-7
Number of subjects	n, *n*
Selected ion-monitoring	SIM

Solid phase extraction	SPE
Standard deviation	SD
Standard error	SE
Subcutaneous	sc
Vesicular-coagulating	VC

Units of Measurement

Centi	c
Curie	Ci
Degrees Celsius	°C
Disintegrations per minute	DPM, dpm
Grams	g
Gravitation force	*g*
Height	h
Hours	h
International unit	IU
Kilo	k
Length	l
Litres	L, l
Mega	M
Metres	m

Micro	µ
Milli	m
Minutes	min
Molar	M
Mole	mol
Nano	n
Ohm	Ω
Pico	p
Potential of hydrogen	pH
Seconds	s
Width	w

Chapter 1

General Introduction

The endocrine system

The endocrine system represents a collection of glands within an organism. These glands secrete hormones that enter circulation, travel throughout the body, and regulate functions of distant target organs (Nelson and Kriegsfeld 2017). In mammals, the major glands of the endocrine system include the pineal gland, pituitary gland, adrenal gland, pancreas, thyroid gland, ovaries, and testes. Secondary endocrine functions are features of other organs, including the liver, kidney, stomach, heart, and bone. The hypothalamus, located adjacent to the pituitary gland, acts as the neural control center of endocrine signaling.

The endocrine system utilizes numerous hormones to govern many broad functions. These functions include growth, development, reproduction, digestion, metabolism, behaviour, and maintaining homeostasis in response to changing environments (Nelson and Kriegsfeld 2017). Secreted hormones accomplish this by acting on receptors, either membrane-bound or intracellular, in target tissues to initiate downstream responses (Walters 1985). Proper functioning of all glands and hormones is a feature of healthy individuals. This thesis will focus on sex steroid hormones given their extensive study in the field of endocrine disruption by environmental toxicants (Gore et al. 2015).

Sex steroid hormones

Sex steroid hormones include androgens, estrogens, and progestogens. Sex steroids are small molecules that are metabolically derived from cholesterol through a

series of enzymatic transformations (Miller 1988; Payne and Hales 2004). Sex steroids are largely produced in the ovaries, testes, and adrenal glands, but they can also be synthesized in small quantities in the liver, adipose, and elsewhere. These small lipophilic molecules readily travel throughout the body, efficiently crossing cell membranes unassisted and transported in blood by carrier proteins, known as sex hormone-binding globulins (Anderson 1974). Sex steroid hormones broadly govern sexual differentiation, sexual development, sexual behaviour, reproduction, fertility, and pregnancy, and they are also involved in other processes such as immune function, learning, and memory (Ruiz-Cortés 2012).

The physiological, behavioural, and other impacts of sex steroids are largely accomplished via binding to receptors in target tissues (Walters 1985). Estrogens, such as estrone (E1), 17β-estradiol (E2), and estriol (E3), activate estrogen receptors (ER). Androgens, such as testosterone and dihydrotestosterone, activate androgen receptors, and progesterone (P4) activates progestogen receptors. Binding results in the formation of a receptor-ligand complex that can upregulate or downregulate gene transcription (Walters 1985). Depending upon the specific sex steroid acting as the ligand, signaling can be highly potent and prolonged, inducing significant changes in gene transcription and protein synthesis lasting from hours to weeks (Ruiz-Cortés 2012).

Given the potent impacts of sex steroids, it is critical that their activity be tightly controlled. This can be accomplished through several mechanisms, including regulation of receptor expression, cofactors involved in transcription, and other molecular messengers within cells (McDonnell and Norris 2002). Perhaps the most direct

mechanism of controlling sex steroid activity is via regulation of biologically active (unconjugated) sex steroid concentrations. Concentrations of sex steroids in the body are tightly regulated by several enzymes, including 3β-hydroxysteroid dehydrogenase (3β-HSD), 11β-hydroxysteroid dehydrogenase (11β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 17α-hydroxylase/17,20-lyase, aromatase (CYP19A1), steroid sulfatase (STS), sulfotransferase (SULT), and UDP-glucuronosyltransferase (UGT) (Hanukoglu 1992; Payne and Hales 2004). Some of these enzymes are responsible for metabolizing sex steroids into their conjugated forms, which cannot bind steroid receptors and are therefore considered biologically inactive.

Congenital abnormalities can result in reduced efficacy of one or more of these enzymes from birth, thereby disrupting steroid concentrations and producing an array of human disorders (White 1994). Androgen excess in females, known as hyperandrogenism, is associated with elevated risk of type 2 diabetes, obesity, and polycystic ovarian syndrome (Azziz et al. 2006). Androgen deficiency in males, known as hypoandrogenism, can lead to reduced libido, gonad size, infertility, headaches, and depression (Bhasin et al. 2010). P4 deficiency in females can also lead to reduced libido, uterine bleeding, and infertility (Csapo et al. 1974). Disruption of androgen and P4 signaling can be problematic; however, the studies conducted in this thesis primarily focus on actions of estrogens and estrogenic chemicals.

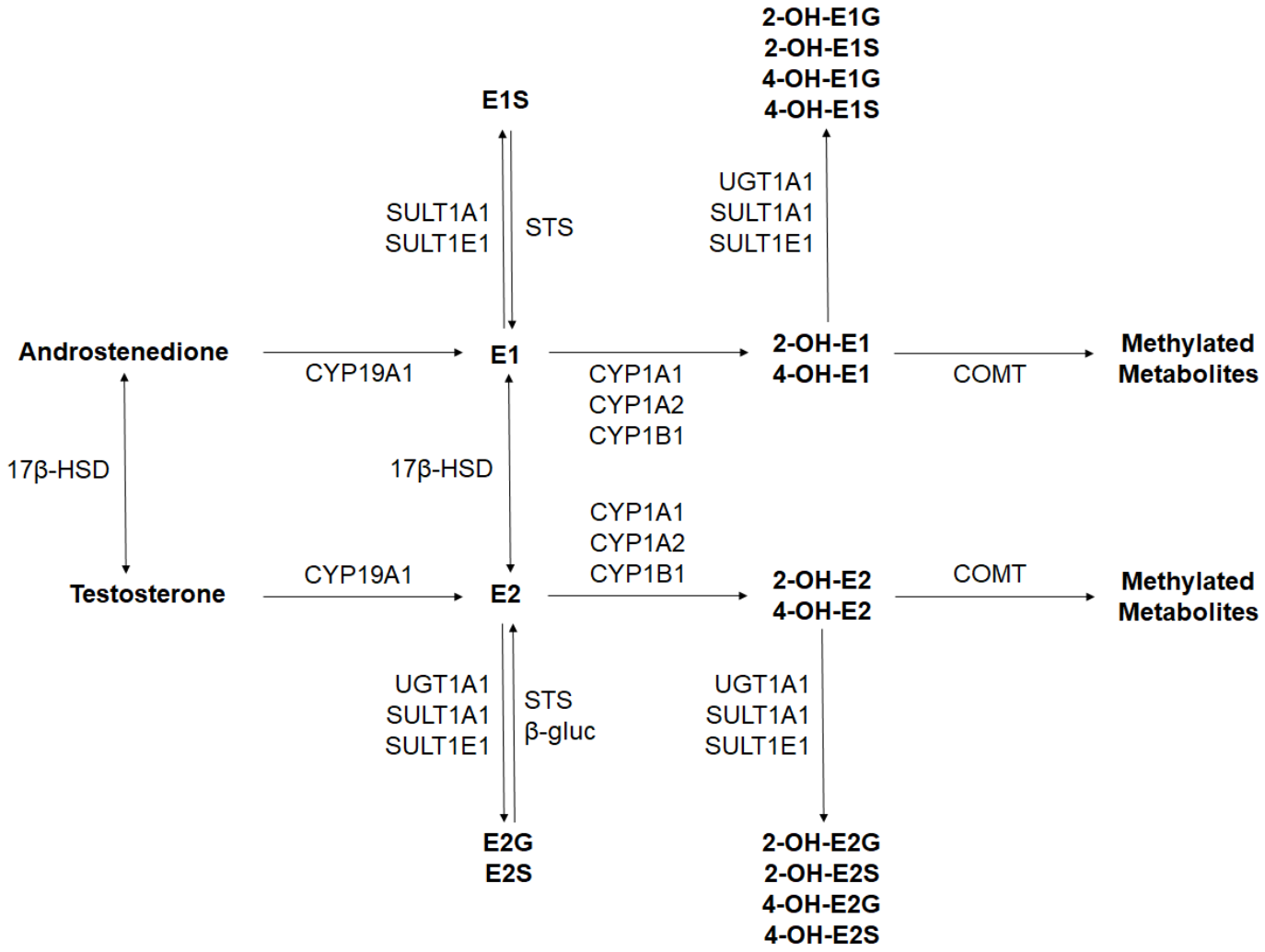
Estrogen function and regulation

Estrogens are the smallest of the sex steroid hormones, located at the end of the steroid biosynthetic pathway (Dumas and Diorio 2011; Payne and Hales 2004). Critical for estrogen production is the cleavage of a methyl group from an androgen precursor, converting either androstenedione to E1 or testosterone to E2 (Fig. 1). The enzyme responsible for this step is CYP19A1 (Dumas and Diorio 2011; Payne and Hales 2004). E3 is the major circulating estrogen during pregnancy and is produced in the liver and placenta (Kota et al. 2013). E1, E2, and E3 are biologically active and can bind to ER, with E2 showing the highest affinity and therefore being considered the most potent natural estrogen (Kuiper et al. 1997).

Conventional ERα and ERβ are intracellular receptors that dimerize and localize into the nucleus upon ligand binding. The ligand-receptor complex interacts with DNA sequences known as estrogen response elements to upregulate or downregulate gene transcription (Carroll et al. 2006; Klinge 2001). Whereas these effects occur on the order of hours to days, more rapid estrogen actions, on the order of seconds to minutes, are accomplished by membrane-associated receptors. One such membrane-associated receptor is G protein-coupled estrogen receptor 1 (GPER) (Barton et al. 2017; Filardo and Thomas 2005; Prossnitz et al. 2008). Unlike ERα and ERβ, GPER does not localize into the nucleus and directly bind DNA. Rather, signaling is achieved through intracellular second messengers, including adenylyl cyclase and protein kinases (Filardo and Thomas 2005; Prossnitz et al. 2008).

Concentrations of biologically active estrogens in circulation and tissues are strictly maintained by actions of several enzymes (Fig. 1). Estrogen conjugates and metabolites cannot bind ER, but instead function as storage reservoirs for estrogens in the body or prepare estrogens for excretion by increasing their hydrophilicity (Reed et al. 2005). Such conjugates include estrone sulfate (E1S) and estradiol sulfate (E2S) produced via interaction with SULT1A1 or SULT1E1 (Dumas and Diorio 2011). Estradiol is glucuronidated by UGT1A1 to form estradiol glucuronide (E2G). Sulfated and glucuronidated estrogens can be converted back into their unconjugated, bioactive forms via STS and β-glucuronidase (β-gluc) respectively. Hydroxylated metabolites include 2-OH-E1, 4-OH-E1, 2-OH-E2, and 4-OH-E2 and are produced via interaction with CYP1A1, CYP1A2, or CYP1B1 (Dumas and Diorio 2011). These hydroxylated metabolites can be sulfated or glucuronidated via interaction with SULT or UGT, respectively, or can be methylated by catechol-*O*-methyltransferase (COMT) (Dumas and Diorio 2011).

Fig. 1 Synthesis and metabolism of estrogens. Adapted from Dumas and Diorio (2011).



Strict regulation of estrogen concentrations and activity is of partcular importance given the role of estrogens in critical biological functions, such as pregnancy, and human diseases, such as cancer. Administration of small quantities of exogenous E2 around the time of intrauterine implantation of fertilized ova (blastocysts) can terminate pregnancy in mice (deCatanzaro et al. 2001). Hyperstimulation of the ovaries causing elevations in endogenous E2 can likewise disrupt blastocyst implantation in mice (Fossum et al. 1989). Women with a high serum E2:P4 ratio were less likely to become pregnant via *in vitro* fertilization (Gidley-Baird et al. 1986). In addition to blocking implantation, high estrogen levels can also directly damage the developing embryo (Valbuena et al. 2001).

Small but persistent elevations in estrogens are also implicated in hormone-dependent cancers. Tumour growth is stimulated by estrogens, as the estrogen-ER complex can upregulate factors that cause the cell to enter the cell cycle and undergo proliferation (Ciocca and Fanelli 1997). Hormone-dependent cancers are most prevalent in post-menopausal women; increased estrogen activity from hormone-replacement therapy is associated with increased risk of breast, endometrial, and ovarian cancer (Million Women Study Collaborators 2003, 2005, 2007). Therapeutic targets to combat hormone-dependent cancers include enzymes responsible for estrogen production, such as CYP19A1 and STS (Chumsri et al. 2011; Maltais and Poirier 2011).

Endocrine-disrupting chemicals

The Endocrine Society defines an endocrine-disrupting chemical (EDC) as “an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormonal

action" (Gore et al. 2015; Zoeller et al. 2012). The U.S. Environmental Protection Agency (EPA) maintains a universe of approximately 10,000 unique chemicals as part of the Endocrine Disruptor Screening Program (EDSP) (U.S. EPA 2012a, 2012b, 2017). Chemicals included in the EDSP include environmental and drinking water contaminants, pesticide active ingredients, and inert substances (U.S. EPA 2012b). These chemicals are being screened for their potential to disrupt estrogen, androgen, and/or thyroid activities.

One of the most well-studied EDCs is bisphenol A (BPA), a monomer of polycarbonate plastics and epoxy resins. Concern surrounding BPA as an EDC has led to its replacement with other bisphenol analogues, such as bisphenol S, bisphenol F, and bisphenol AF (Chen et al. 2016). Despite this, BPA is still present in many consumer products, including plastic food storage containers, canned foods and beverages, and thermal receipt paper (Ehrlich et al. 2014; Vandenberg et al. 2007). BPA can leach from these and other products due to incomplete polymerization and exposure to heat (Al-Hiyasat et al. 2004; Brotons et al. 1995; Krishnan et al. 1993). Leached BPA can be ingested, inhaled, and absorbed through the skin; however, ingestion from contaminated foods and beverages accounts for 85–95% of total BPA exposure in human adults (EFSA 2015). According to the 2011–2012 U.S. National Health and Nutrition Examination Survey (NHANES), BPA was detected in 89.1% of individuals 6 years of age and older, with total urinary concentrations ranging from 0.4 µg/L to 179.0 µg/L. The geometric mean and 95th percentile urinary BPA concentrations were 1.5 µg/L and 9.4 µg/L respectively (CDC 2015).

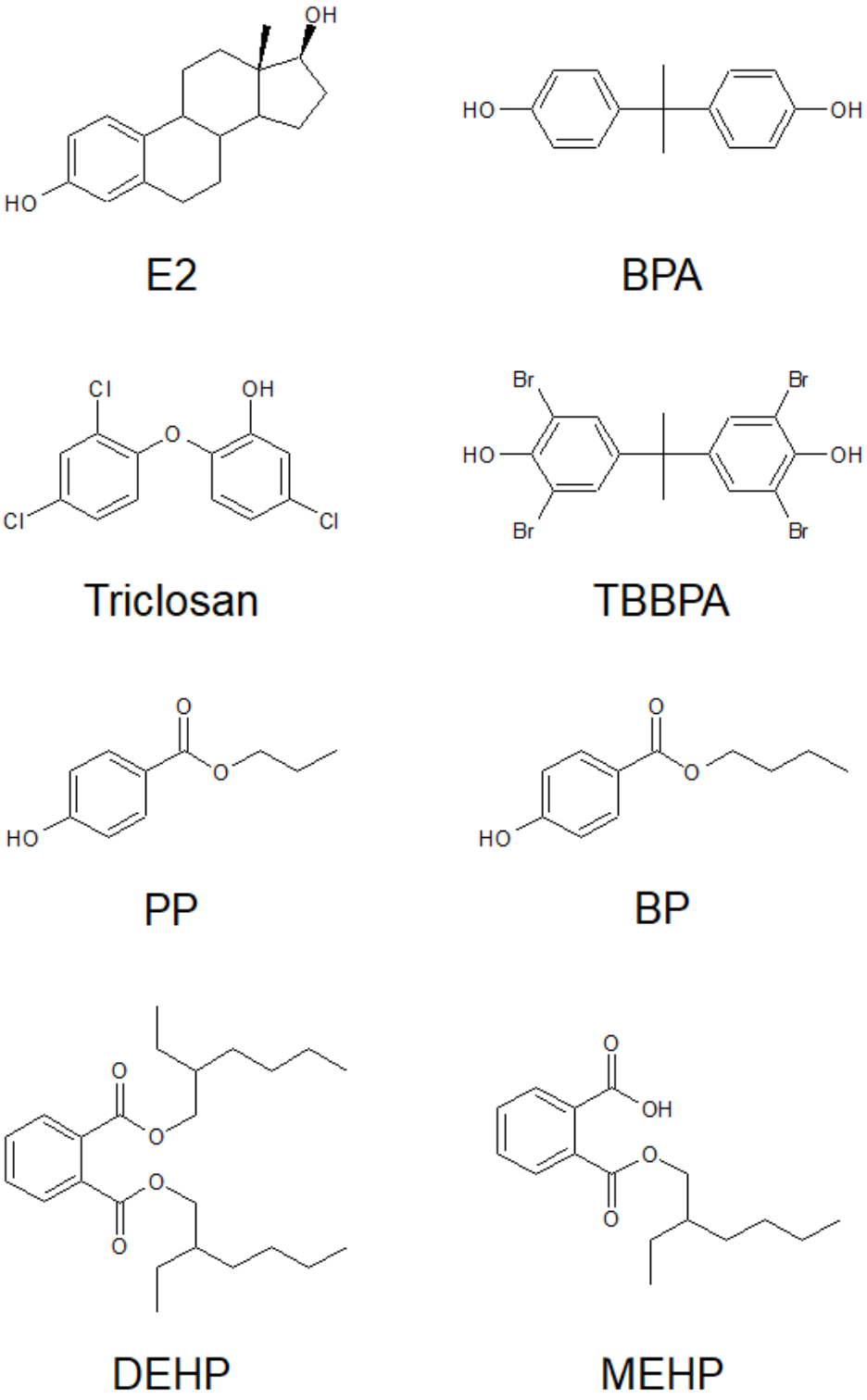
BPA can bind to conventional nuclear ERα and ERβ with approximately 3–4 orders of magnitude lower affinity than that of E2 (Kuiper et al. 1997; Molina-Molina et al. 2013). BPA also shows high affinity for membrane-bound GPER with approximately 1–2 orders of magnitude lower affinity than that of E2 (Dong et al. 2011; Thomas and Dong 2006). Furthermore, BPA shows high affinity for estrogen-related receptor γ (ERRγ), which has no known endogenous ligands (Matsushima et al. 2007; Okada et al. 2008). As a result of its interaction with receptors, BPA has well-established estrogenic effects (Rochester 2013; Seachrist et al. 2016; Ziv-Gal and Flaws 2016). BPA is broadly associated with disruptions in female reproductive organ morphology, estrous cycling, and blastocyst implantation (Berger et al. 2008, 2010; Ziv-Gal and Flaws 2016). BPA may also promote breast and prostate tumour growth (Seachrist et al. 2016) and is implicated in developmental and behavioural abnormalities in children (Rochester 2013).

The studies included in this thesis examined the capacity of certain EDCs to modulate concentrations of BPA and natural E2 *in vivo*. Given the large number of chemicals included in the EDSP, I established criteria for selection of EDCs to be studied. The criteria for selection were as follows: [1] the chemical must be present in consumer products to which people are frequently exposed; [2] the chemical must be detectable in a considerable portion of the population or, in the absence of large-scale biomonitoring data, likely to be present in a considerable portion of the population; [3] the chemical must have evidence of estrogenic and/or antiestrogenic effects *in vivo*; and [4] the chemical, or one of its major metabolites, must have *in silico* or *in vitro* evidence of interaction with enzymes involved in the metabolism of BPA and E2. The five

chemicals selected for inclusion in studies conducted as part of this thesis were triclosan, butyl paraben (BP), propyl paraben (PP), tetrabromobisphenol A (TBBPA), and di(2-ethylhexyl) phthalate (DEHP) (Fig. 2).

Triclosan is a synthetic biocide that inhibits bacterial fatty acid synthesis via interaction with enoyl-acyl carrier protein reductases (Heath et al. 1999). Given its broad-spectrum antibacterial properties, triclosan is added to many consumer and personal care products, including soaps, cosmetics, toothpastes, household cleaners, and clothing (Fang et al. 2010). Triclosan is present in a majority of the U.S population with highly variable concentrations. According to the 2011–2012 NHANES, 72% of human urine samples contained triclosan, with concentrations ranging from 2.3 to 3,830 μg/L and a mean of 12.5 μg/L (Han et al. 2016). Triclosan has also been detected in human blood serum (Allmyr et al. 2008; Geens et al. 2009), blood plasma (Allmyr et al. 2006), breast milk (Allmyr et al. 2006), adipose (Geens et al. 2012), and liver tissue (Geens et al. 2012). Triclosan has estrogenic effects in rodent uterotrophic assays (Jung et al. 2012; Louis et al. 2013; Stoker et al. 2010). High urinary triclosan levels in humans are associated with reduced intrauterine implantation rate during *in vitro* fertilization procedures (Hua et al. 2017) and reduced weight, length, and gestational age of infants at birth (Etzel et al. 2017). Estrogenic effects of triclosan may arise from interactions with ER (Gee et al. 2008; Kim et al. 2014); however, triclosan can also disrupt metabolism of E2 and BPA (James et al. 2010, 2015; Wang et al. 2004).

Fig. 2 Chemical structures of E2, BPA, triclosan, TBBPA, BP, PP, DEHP, and MEHP.



Parabens (*p*-hydroxybenzoic acid esters) are antimicrobial preservatives added to personal care products, pharmaceuticals, foods, and beverages (Andersen 2008). According to the 2005–2006 NHANES, PP and BP were detected in 92.7% and 47% of human urine samples, with concentrations ranging from 0.2 to 7,210 μg/L and 0.2 to 1,240 μg/L respectively (Calafat et al. 2010). Parabens have also been detected in human blood serum (Frederiksen et al. 2011; Ye et al. 2008b), breast milk (Ye et al. 2008a), breast tumour tissue (Darbre et al. 2004), placental tissue (Jiménez-Díaz et al. 2011), and seminal plasma (Frederiksen et al. 2011). Parabens have estrogenic effects in rodent uterotrophic assays (Lemini et al. 2004; Routledge et al. 1998), can promote estrogenic histological changes in the uterus (Lemini et al. 2004), and can disrupt male reproductive parameters (Garcia et al. 2017; Oishi 2002a, 2002b; Zhang et al. 2016). Parabens are known ER agonists (Blair et al. 2000; Byford et al. 2002; Gomez et al. 2005; Mansouri et al. 2016; Miller et al. 2001; Watanabe et al. 2013), but their estrogenic effects could also be due to inhibition of UGT, SULT, and/or CYP activities (Abbas et al. 2010; Ozaki et al. 2016; Prusakiewicz et al. 2007), thus disrupting natural estrogen homeostasis.

TBBPA is a flame retardant commonly incorporated into printed circuit boards and plastic housings of consumer electronics (Colnot et al. 2014; Shaw et al. 2014). Although TBBPA has not been assessed in NHANES, one study of 140 healthy adults in China found that 89% of urine samples contained detectable TBBPA concentrations at up to 88.4 μg/g creatinine (Ho et al. 2017). TBBPA has also been detected in human serum (Cariou et al. 2008; Fujii et al. 2014), plasma (Ho et al. 2017), breast milk (Abdallah and Harrad 2011; Fujii et al. 2014; Nakao et al. 2015), and adipose (Cariou et al. 2008;

Johnson-Restrepo et al. 2008). In addition to estrogenic effects in rodent uterotrophic assays (Kitamura et al. 2005), TBBPA also increased incidence of uterine tumours in rats as part of a 2-year study by the U.S. National Toxicology Program (NTP) (NTP 2014). Some studies have suggested that TBBPA is an ER agonist (Li et al. 2010; Olsen et al. 2003; Suzuki et al. 2013), whereas others showed no such evidence (Dorosh et al. 2011; Hamers et al. 2006; Lee et al. 2012; Meerts et al. 2001; Miller et al. 2001; Molina-Molina et al. 2013; Riu et al. 2011a, 2011b). Rather, TBBPA could disrupt natural estrogen homeostasis (Wikoff et al. 2016) by inhibiting UGT, SULT, HSD, and/or CYP activities (Ames 2013; Gosavi et al. 2013; Hamers et al. 2006; Harju et al. 2007; Kester et al. 2002; NIH 2010).

DEHP is a plasticizer commonly used in products made of polyvinyl chloride, including flooring, medical devices, and food packaging (Mariana et al. 2016; Zarean et al. 2016). DEHP has estrogenic effects in rodent uterotrophic assays (Zacharewski et al. 1998), disrupted hypothalamic-uterine signaling via gonadotropin-releasing hormone (Liu et al. 2016), and reduced endometrial receptivity and blastocyst implantation in mice (Li et al. 2012). There is conflicting evidence regarding the capacity of DEHP to act as an ER agonist (Blair et al. 2000; Mansouri et al. 2016; Takeuchi et al. 2005). However, a single dose of 3–18 mg DEHP can elevate concentrations of dietary ^{14}C-BPA in male and female mice (Borman et al. 2017). This finding could be attributed to inhibition of BPA conjugation by mono(2-ethylhexyl) phthalate (MEHP), a major metabolite of DEHP (Hanioka et al. 2012, 2016). Other metabolites of DEHP include mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and

mono(2-ethyl-5-carboxypentyl) phthalate (MECPP). Whereas DEHP was not directly assessed in the NHANES 2009–2010 project, MEHP, MEHHP, MEOHP, and MECPP were detected in 56, 98, 96, and 100% of human urine samples with means of 2.0, 13.8, 8.7, and 20.4 μg/L, respectively (Zota et al. 2014).

Research objectives and overview

The first objective of this thesis was to establish the distribution of BPA in blood serum and tissues of rodents following dietary exposure at doses lower than previously studied. In research reported in **Chapter 2**, I measured the distribution of BPA in blood serum, neural, and peripheral tissues of female rats and mice following single or repeated dietary exposure at doses ranging from 0.5–50 μg/kg. I hypothesized that BPA would localize to the reproductive organs of females following a single dietary dose and that BPA would also accumulate there following repeated daily doses. This is consistent with high ER expression in reproductive organs (Kuiper et al. 1997) and also with previous studies examining the distribution of BPA in rodents at much higher doses (Kim et al. 2004; Kurebayashi et al. 2005).

The remaining objectives of this thesis were to assess the acute impact of EDCs, administered either alone or as a mixture, on concentrations of dietary BPA and natural E2 in rodents. Given evidence that the EDCs described above compete for enzymes involved in the metabolism of BPA and E2, I hypothesized that exposure to these chemicals would elevate concentrations of BPA in blood serum and tissues and E2 in urine of mice. Whereas I expected that higher doses of a single chemical would elevate

BPA and E2 concentrations, I also hypothesized that lower doses of multiple chemicals would be sufficient to achieve similar effects. I implemented a novel protocol that involved a single subcutaneous injection of one or multiple chemicals followed shortly after by dietary administration of ^{14}C-BPA in peanut butter. I then measured radioactivity in blood serum and tissues via liquid scintillation counting and E2 in urine via enzyme-linked immunosorbent assay (ELISA). These techniques permitted rapid, low-cost assessment of the capacity of EDCs to interact and disrupt natural estrogen homeostasis *in vivo*.

Chapter 3 reports assessment of the impact of 0.2–18 mg triclosan on the distribution of 5–50 μg/kg ^{14}C-BPA in female and male mice. **Chapter 4** provides evidence of the impact of 0.6–2 mg triclosan on the distribution of ^{3}H-E2 in cycling and recently inseminated female mice, as well as the impact of 1–2 mg triclosan on urinary concentrations of natural E2 in urine of cycling and recently inseminated female mice. **Chapter 5** reports research showing the impact of 1–9 mg PP or BP on the distribution of 50 μg/kg ^{14}C-BPA, as well as the impact of 3 mg PP or BP on natural E2 in urine of female and male mice. **Chapter 6** gives evidence of the impact of 1–27 mg TBBPA on the distribution of 50 μg/kg ^{14}C-BPA, as well as the impact of 1 mg TBBPA on natural E2 in urine of female and male mice. I also measured additive effects of TBBPA and triclosan on the distribution of 50 μg/kg ^{14}C-BPA by administering sub-threshold (0.33 mg) doses of TBBPA and triclosan, given alone or in combination. **Chapter 7** reports assessment of potential interactions among a mixture of the above 4 EDCs, as well as DEHP. To do so I administered 0.1 or 0.5 mg triclosan, PP, BP, TBBPA, DEHP, or a 0.5

mg mixture containing 0.1 mg of all five chemicals, on the distribution of 50 µg/kg ^{14}C-BPA. I also measured the impact of the 0.5 mg mixture containing 0.1 mg of all five chemicals on natural E2 in urine of female and male mice. In **Chapter 8**, I summarize the findings of Chapters 2–7, discuss the importance of these findings in the context of human health and regulatory decision-making, and suggest areas of future research.

Chapter 2: Pollock T, deCatanzaro D 2014 Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses *Reproductive Toxicology* **49** 145–154.

Abstract: This research examined the distribution of low dietary doses of bisphenol A (BPA). When female rats received 50 µg/kg ^{14}C-BPA orally, radioactivity was distributed throughout the body, with especial presence in the uterus. Pre-treatment with estradiol or the estrogen antagonist ICI 182,780 significantly reduced radioactivity in the uterus. The majority of BPA at the uterus was determined to be aglycone (receptor-active) via GC-MS. Subsequently, mice given 0.5, 5, or 50 µg/kg ^{14}C-BPA showed more radioactivity in the uterus than in other non-metabolic tissues. When female mice received 1, 7, or 28 daily doses of 50 µg/kg ^{14}C-BPA, then were measured 24 hours after the last dose, significantly more radioactivity was detected in the uterus, liver, and kidney following repeated doses. Collectively, these data provide evidence for the *in vivo* interaction of BPA with estrogen receptors. They also indicate elevated presence of BPA in reproductive tissues after repeated low doses.

Chapter 3: Pollock T, Tang B, deCatanzaro D 2014 Triclosan exacerbates the presence of ^{14}C-bisphenol A in tissues of female and male mice *Toxicology and Applied Pharmacology* **278** 116–123.

Abstract: Current human generations are commonly exposed to both triclosan (TCS), an antimicrobial agent, and bisphenol A (BPA), the monomer of polycarbonate plastics and epoxies. Both are readily absorbed into circulation and found distributed among diverse tissues. Potential interactions between TCS and BPA are largely unstudied. We investigated whether TCS exposure affects the distribution of ingested ^{14}C-BPA in select tissues. CF-1 mice were each subcutaneously injected with TCS then orally administered 50 µg/kg ^{14}C-BPA. Females received 0, 0.2, 0.6, 1, 2, or 18 mg TCS (equivalent respectively to 0, 6.3, 16.9, 30.1, 60.5, and 558.9 mg/kg). Males received 0, 0.2, 2, or 18 mg TCS (equivalent respectively to 0, 5.3, 53.4, and 415.0 mg/kg). Levels of radioactivity were measured through liquid scintillation counting in blood serum and brain, reproductive, and other tissues. Significantly elevated levels of radioactivity were observed following combined TCS and ^{14}C-BPA administration, with minimally effective TCS doses being tissue-dependent (Females: lungs, 0.6 mg; uterus, 1 mg; heart, muscle, ovaries, and serum, 18 mg. Males: serum, 0.2 mg; epididymides, 2 mg). Subsequently, we found that 2 or 6 mg TCS increased radioactivity in the ovaries and serum of females orally given only 5 µg/kg ^{14}C-BPA. These data indicate that TCS can interact with BPA *in vivo*, magnifying its presence in certain tissues and serum. The data are consistent with evidence that TCS utilizes enzymes that are critical for metabolism and excretion of

BPA. Further research should investigate the mechanisms through which these two chemicals interact at environmentally-relevant doses.

Chapter 4: Pollock T, Greville LJ, Tang B, deCatanzaro D 2016 Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice *Reproductive Toxicology* **65** 394–401.

Abstract: Triclosan, an antimicrobial agent added to personal care products, can modulate estrogenic actions. We investigated whether triclosan affects concentrations of exogenous and endogenous estradiol. Female mice were given injections of triclosan followed by 1 µCi tritium-labeled estradiol. Mice given daily 2-mg triclosan doses (57.9 mg/kg/dose) showed significantly elevated radioactivity in tissues and serum compared to controls. A single dose of 1 or 2 mg triclosan increased radioactivity in the uterus in both cycling and peri-implantation females. We also measured natural urinary estradiol at 2–12 h following triclosan injection. Unconjugated estradiol was significantly elevated for several hours following 1 or 2 mg of triclosan. These data are consistent with evidence that triclosan inhibits sulfonation of estrogens by interacting with sulfotransferases, preventing metabolism of these steroids into biologically inactive forms. Elevation of estrogen concentrations by triclosan is potentially relevant to anti-reproductive and carcinogenic actions of excessive estrogen activity.

Chapter 5: Pollock T, Weaver RE, Ghasemi R, deCatanzaro D 2017 Butyl paraben and propyl paraben modulate bisphenol A and estradiol concentrations in female and male mice *Toxicology and Applied Pharmacology* **325** 18–24.

Abstract: People are routinely exposed to the antimicrobial preservatives butyl paraben (BP) and propyl paraben (PP), as well as the monomer of polycarbonate plastics, bisphenol A (BPA). These chemicals are reliably detected in human urine and potentially interact. We investigated whether BP or PP exposure can modulate the concentrations of ^{14}C-BPA and 17β-estradiol (E_2). Female and male CF1 mice were each given a subcutaneous injection of oil containing 0 (vehicle), 1, 3, or 9 mg BP or PP, then given a dietary supplement containing 50 µg/kg ^{14}C-BPA. Radioactivity was measured in tissues through liquid scintillation counting. Significantly elevated ^{14}C-BPA concentrations were observed following BP treatment in blood serum of both sexes, as well as the lungs, uterus, and ovaries of females and the testes and epididymides of males. Treatment with PP significantly elevated ^{14}C-BPA concentrations in the uterus only. In another experiment, female and male CF1 mice were each injected with vehicle, 3 mg BP, or 3 mg PP, and E_2 was measured in urine 2–12 h later. Whereas PP did not affect E_2, BP significantly elevated E_2 6–10 h after injection in females and 8 h after injection in males. These data indicate that BP and PP can alter the pharmacokinetics of BPA *in vivo*, and that BP can modulate E_2 concentrations. These results are consistent with evidence that parabens inhibit enzymes that are critical for BPA and E_2 metabolism, and demonstrate the importance of considering concurrent exposure to multiple chemicals when determining regulatory exposure limits.

Chapter 6: Pollock T, Mantella L, Reali V, deCatanzaro D 2017 Influence of tetrabromobisphenol A, with or without concurrent triclosan, upon bisphenol A and estradiol concentrations in mice *Environmental Health Perspectives* **in press**.

Abstract: *Background:* Humans are commonly exposed to multiple environmental chemicals, including tetrabromobisphenol A (TBBPA; a flame retardant), triclosan (an antimicrobial agent), and bisphenol A (BPA; polycarbonate plastics). These chemicals are readily absorbed and potentially interact. *Objectives:* We sought to determine whether TBBPA, given alone or in combination with triclosan, can modulate the concentrations of BPA and 17β-estradiol (E2). *Methods:* Female and male CF1 mice were each given a subcutaneous injection of 0–27 mg TBBPA, with or without concurrent 0.33 mg triclosan, followed by dietary administration of 50 µg/kg bodyweight ^{14}C-BPA. Radioactivity was measured in blood serum and tissues through liquid scintillation counting. In subsequent experiments, female and male CF1 mice were each given a subcutaneous injection of 0 or 1 mg TBBPA, and E2 was measured in urine 2–12 h after injection. *Results:* Doses as low as 1 mg TBBPA significantly elevated ^{14}C-BPA concentrations in the uterus and ovaries of females; the testes, epididymides, vesicular-coagulating glands, and preputial glands of males; and blood serum, heart, lungs, and kidneys of both sexes. They also elevated urinary E2 concentrations. Lower doses of TBBPA or triclosan that had no effect on their own elevated ^{14}C-BPA concentrations when the two substances were given concurrently. *Conclusions:* These data indicate that

TBBPA, triclosan, and BPA interact *in vivo,* consistent with evidence that TBBPA and triclosan inhibit enzymes that are critical for BPA and E2 metabolism.

Chapter 7: Pollock T, Weaver RE, Ghasemi R, deCatanzaro D A mixture of five endocrine-disrupting chemicals modulates concentrations of bisphenol A and estradiol in mice.

Abstract: Most people in developed countries are exposed to multiple endocrine-disrupting synthetic chemicals. We previously showed that a single dose of triclosan, tetrabromobisphenol A (TBBPA), butyl paraben, propyl paraben, or di(2-ethylhexyl) phthalate elevated concentrations of bisphenol A (BPA) in mice. Here we investigated whether concurrent exposure to lower doses of these five chemicals could modulate concentrations of bisphenol A (BPA) or the natural estrogen, 17β-estradiol (E2). CF1 mice were injected subcutaneously with 0.1 or 0.5 mg of one chemical, or a 0.5 mg mixture containing 0.1 mg of each of all five chemicals, then given a dietary supplement containing 50 μg/kg 14C-BPA. The mixture elevated 14C-BPA concentrations in the lungs, muscle, uterus, ovaries, kidney, and blood serum of female mice. When administered alone, triclosan and TBBPA elevated 14C-BPA concentrations in the uterus, ovaries, and blood serum. In another experiment, CF1 mice were injected subcutaneously with the 0.5 mg mixture containing 0.1 mg of each chemical, then E2 was measured in urine 2–12 h later. The mixture elevated E2 at 8 h after injection in female mice. No treatments significantly altered concentrations of 14C-BPA or E2 in male mice. These data show that these endocrine-disrupting chemicals interact in vivo, magnifying one

another's effects, consistent with inhibition of enzymes that are critical for estrogen metabolism. These findings highlight the importance of considering exposure to multiple chemicals when assessing health outcomes and determining regulatory exposure limits.

Chapter 2

Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses

Tyler Pollock and Denys deCatanzaro (2014)

Reproductive Toxicology, 49, 145–154

Authors' Contributions

Tyler Pollock: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

Denys deCatanzaro: Assistance with experimental design, data analysis, manuscript editing and preparation.

Abstract

This research examined the distribution of low dietary doses of bisphenol A (BPA). When female rats received 50 µg/kg ^{14}C-BPA orally, radioactivity was distributed throughout the body, with especial presence in the uterus. Pre-treatment with estradiol or the estrogen antagonist ICI 182,780 significantly reduced radioactivity in the uterus. The majority of BPA at the uterus was determined to be aglycone (receptor-active) via GC-MS. Subsequently, mice given 0.5, 5, or 50 µg/kg ^{14}C-BPA showed more radioactivity in the uterus than in other non-metabolic tissues. When female mice received 1, 7, or 28 daily doses of 50 µg/kg ^{14}C-BPA, then were measured 24 hours after the last dose, significantly more radioactivity was detected in the uterus, liver, and kidney following repeated doses. Collectively, these data provide evidence for the *in vivo* interaction of BPA with estrogen receptors. They also indicate elevated presence of BPA in reproductive tissues after repeated low doses.

1. Introduction

Bisphenol A (BPA) is a known endocrine disruptor used as a monomer in the production of polycarbonate plastics and epoxy resins. BPA is found in many household, commercial, and medical products, including drinking bottles, food storage containers, water pipes, dental sealants, thermal paper, and electronics [1]. Due to incomplete polymerization during the manufacturing process and depolymerization caused by heat, BPA can leach from products into the surrounding environment [2–6]. Given the abundant production, use, and disposal of products containing BPA, it has been detected in humans, wildlife, water treatment plants, and landfills [7–10]. Several studies have measured BPA levels in adult human serum ranging from undetectable to 1.6 ng/ml [11–13] and in urine from undetectable to 149.0 ng/ml [14–17]. Over 90% of human urine samples from the United States contain detectable BPA levels, suggesting widespread exposure [14,17], and similar data have been found in other countries [18].

Ingestion of BPA through contaminated food and beverages is the primary route of human exposure [1,8,19,20]. Adult daily dietary intake of BPA is estimated at 0.1–0.37 µg/kg [21,22]. Accordingly, animal models involving BPA administered orally in food represent human exposure most realistically. Compared to subcutaneous, intraperitoneal, and intravenous injection, there is efficient first-pass hepatic elimination of BPA following intragastric exposure via gavage [23–25]. However, ingestion of BPA through dietary sources results in attenuated first-pass hepatic elimination and elevated levels of BPA in serum compared to bolus intragastric administration [26]. Some evidence indicates that dietary BPA exposure results in plasma BPA concentrations

comparable to those measured following subcutaneous injection [27]. Inside the body, BPA is metabolized by the enzymes UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT), which are constitutively expressed in the liver of rodents and humans [28–30]. Interaction of BPA with UGT or SULT produces one of several conjugated forms, with BPA monoglucuronide being the primary form in both rats and humans [25,31].

Despite metabolism and elimination of BPA with varying efficiency dependent upon the route of exposure [25,32], some portion of the chemical remains unconjugated [33]. Aglycone (unconjugated) BPA interacts with both estrogen receptors (ER) α and β [34–36]. In addition to conventional nuclear ER, aglycone BPA has high affinity for two membrane-bound ER, GPR30 [37] and mERα [38], as well as an orphan nuclear estrogen-related receptor, ERRγ [39,40]. Although conjugated forms of BPA have been considered to be biologically inactive due to their inability to compete with 17β-estradiol (E_2) for binding to ERα and ERβ [41,42], recent evidence suggests that BPA metabolites may interfere with rapid ER signalling through interactions with mERα [43]. BPA is commonly referred to as a weak xenoestrogen, given that it possesses approximately 10,000-fold lower affinity for ERα and ERβ relative to E_2 [44,45]. However, some studies have provided evidence for molecular mechanisms of BPA at low concentrations, capable of producing cellular responses with comparable potency of E_2 [38,46]. The interaction of BPA with non-genomic ER may be implicated in these mechanisms [47]. There are many reports of estrogenic effects of BPA at doses comparable to human exposure levels [48], but such effects have been controversial [49–53].

A number of previous studies have examined the distribution of BPA in tissues of laboratory rodents after a single administration [24,41,54–57]. Intragastric administration to rats in doses ranging from 100 µg/kg to 100 mg/kg ^{14}C-BPA yields radioactivity throughout the body, with highest levels in the liver, kidney, stomach, and intestines [41,55,56], clear presence in reproductive tissues, and much lower levels in the brain [55,56]. Studies of the time course after a single gavage of ^{14}C-BPA indicated that radioactivity largely dissipates within 24 hours, except in the liver and kidney [55,56]. Other studies [24,57] have focused on quantification of aglycone relative to total BPA in various tissues. After i.v. injection of 100 µg/kg BPA, low levels of aglycone BPA have been observed in the liver and serum, and greater levels of the aglycone form in the uterus, ovaries, adipose, brain, and muscles [57].

The purposes of the present study were to determine the disposition of lower oral doses of BPA than previously studied, to examine the potential for binding to receptors, and to investigate the impact of repeated doses on bodily distribution. We first administered a single oral dose of 50 µg/kg ^{14}C-BPA naturalistically and non-invasively to female rats through a food supplement. A subset of rats also received either E_2 or ICI 182,780, a potent nonselective anti-estrogen, to shed light on ER binding. Having observed strong effects at the uterus, we quantified the amount of aglycone versus total BPA there through gas chromatographic-mass spectrometric (GC-MS) analysis. We subsequently employed mice as they are more economical for multiple ^{14}C-BPA administrations, and because analysis of specific brain tissues was not showing substantial radioactivity, thus negating the advantage of using the larger species. We

examined the distribution of lower doses (5 and 0.5 µg/kg) of ^{14}C-BPA in tissues of female mice. Finally, we examined repeated doses of 50 µg/kg ^{14}C-BPA in female mice to determine whether this impacted distribution of radioactivity.

2. Methods

2.1. Animals and housing

Rats aged 6-8 months were of Long-Evans strain and housed in polypropylene cages measuring 44×23×20 cm. Mice aged 4 months were of CF-1 strain and housed in polypropylene cages measuring 28×16×11 cm. All animals were obtained from Charles River (Kingston, NY) and permitted *ad libitum* access to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle, with onset of darkness at 8 AM. This research was approved by the Animal Research Ethics Board of McMaster University, conforming to the standards of the Canadian Council on Animal Care.

2.2. Chemicals and materials

^{14}C-BPA ([ring-[^{14}C](U)]-BPA, in ethanol, 0.1 mCi/ml, 106 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, and Midi-Vial scintillation vials were obtained from PerkinElmer, Waltham, MA. Unlabelled BPA (≥99% purity), 17β-estradiol (≥98% purity), fulvestrant (ICI 182,780, ≥98% purity), toluene (anhydrous, 99.8% purity), methanol (≥99.9% purity), acetonitrile (≥99.9% purity), dichloromethane (anhydrous,

≥99.8% purity), and β-glucuronidase (Type HP-2, from *Helix pomatia*, ≥ 100,000 units/ml) were obtained from Sigma-Aldrich, St. Louis, MO. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, ≥98.5% purity), propylsyringol-d_6 (ACS grade) and pyrene-d_{10} (ACS grade) were obtained from Supelco Analytical, Bellefonte, PA. Glacial acetic acid (≥99.7% purity) and *n*-hexane (≥99% purity) were obtained from Caledon Laboratories, Georgetown, ON. Sodium acetate trihydrate (≥99% purity) was obtained from EMD Chemicals, Gibbstown, NJ. All reagents were of analytical grade unless otherwise specified. Water (18.2 MΩ/cm) was purified using a Milli-Q ultrapure system (Millipore, Billerica, MA).

2.3. Experiment 1: Distribution of ^{14}C-BPA in cycling female rats

On the day prior to start of the experiment, isolated female rats with regular estrous cycles were weighed and randomly assigned to one of four conditions: ^{14}C-BPA, ^{14}C-BPA + E_2, ^{14}C-BPA + ICI 182,780, or contamination control (n=5 per condition; see Table S1). At the onset of darkness on the first and second day, females in the ^{14}C-BPA + ICI 182,780 were injected subcutaneously with 0.10 ml peanut oil containing 300 µg ICI 182,780, whereas those in other conditions were untreated. At the onset of light on the second day, each female was given 1 g peanut butter in a glass dish placed inside its cage. Regular rodent chow was withheld overnight to ensure consumption of peanut butter and prevent dietary neophobia. At the onset of darkness on the third day, all females received a subcutaneous injection of 0.10 ml peanut oil; this contained 20 µg E_2 in the ^{14}C-BPA + E_2 condition and 300 µg ICI 182,780 in the ^{14}C-BPA + ICI 182,780 condition. Three ICI 182,780 injections were given following procedures established elsewhere [58,59]. At 30

min after injection, females in the ^{14}C-BPA, ^{14}C-BPA + E_2, and ^{14}C-BPA + ICI 182,780 conditions were administered 50 µg/kg ^{14}C-BPA in 1 g peanut butter. Females in the control condition were administered the equivalent dose of ethanol in 1 g peanut butter. Water and bedding were removed immediately prior to administration of ^{14}C-BPA or ethanol to prevent contamination of the peanut butter.

At 1 h following administration of ^{14}C-BPA or ethanol, each female was anesthetized with isoflurane and blood was collected via cardiac puncture. The animal was then perfused with 100 ml phosphate-buffered saline. Tissue samples were collected and placed in pre-weighed 8 ml scintillation vials. Tissues taken include a sample of the heart, lung, muscle from the hind leg, abdominal adipose, liver, and a cross-section of the kidney encompassing both the cortex and medulla. Reproductive tissues included a sample of the uterus (medial between the vaginal canal and the oviduct) and both ovaries. Brain areas were taken for the contamination control and ^{14}C-BPA conditions only. Brain areas included a sample of the cerebellum, hypothalamic region (posterior to the optic chiasm and anterior to the pituitary stalk on the ventral brain surface), and amygdaloid region (lateral to the hypothalamus beneath the surface of the cortex). Bilateral sections of the olfactory bulbs, frontal cortex, striatum, and hippocampus were also collected following published procedures [60]. Following tissue collection, vials were re-weighed to determine the wet mass of each sample.

Female tissue samples were homogenized by adding 1 ml SOLVABLE to each vial. Vials were then placed in a 50 °C water bath, swirled after 2 h, and then left in the bath for an additional 2-3 h until completely dissolved. After removal from the bath and

10 min of cooling, 5 ml Ultima Gold scintillation cocktail was added to each vial. Blood samples were centrifuged at 1,500 *g* for 10 min and 10 µl serum was extracted and added to scintillation vials containing 5 ml Ultima Gold. Tissue and serum vials were agitated for 10 min to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber in a TriCarb 2910 TR Liquid Scintillation Analyser (PerkinElmer, Waltham, MA) with a high sensitivity option for 5 min to eliminate background noise in the form of heat and luminescence. Following this interval, radioactivity was measured for 5 min per vial, and final adjusted estimates for the amount of radioactivity per sample in disintegrations per minute (DPM) were automatically calculated by the accompanying Quanta-Smart software package. DPM was standardized to the weight of the sample wet mass as DPM/g tissue, then converted to and reported as equivalent ng BPA/g tissue or ng BPA/ml serum.

2.4. Experiment 2: Distribution of ^{14}C-BPA in inseminated female rats

Given BPA's ability to alter uterine morphology [61] and disrupt intrauterine implantation of fertilized ova [62,63], we replicated Experiment 1 using recently-inseminated female rats, with ^{14}C-BPA intervention around the time of implantation. Sexually naïve female rats were each paired with a male rat aged 6-10 months, and their hindquarters inspected three times daily for the presence of vaginal sperm plugs. Following sperm plug detection, which marked gestation day 0, females were isolated, weighed, and randomly assigned to one of four conditions as in Experiment 1 (n=5 per condition; see Table S1). All other procedures were identical to those of Experiment 1, with days 1–3 described above corresponding to gestation days 1–3.

Table S1 Timing of treatments administered to cycling female rats (Experiment 1) and inseminated female rats (Experiment 2). Oil, E_2, and ICI 182,780 were injected subcutaneously, whereas ethanol and ^{14}C-BPA were given in a dietary supplement of peanut butter (PB). For inseminated female rats (Experiment 2), day 1 corresponded to gestation day 1.

	Day 1 8:00 AM	**Day 2 8:00AM**	**Day 2 6:00PM**	**Day 3 8:00AM**	**Day 3 8:30AM**	**Day 3 9:30AM**
Contamination Control			PB	oil	PB + ethanol	tissues sampled
^{14}C-BPA			PB	oil	PB + ^{14}C-BPA	tissues sampled
^{14}C-BPA + E_2			PB	E_2	PB + ^{14}C-BPA	tissues sampled
^{14}C-BPA + ICI 182,780	ICI 182,780	ICI 182,780	PB	ICI 182,780	PB + ^{14}C-BPA	tissues sampled

2.5. Experiment 3: Quantification of aglycone versus total BPA in the uterus of female rats

2.5.1. Sample collection

Isolated female rats were weighed, given a 1 g peanut butter supplement, and regular rodent chow was withheld overnight. At the onset of darkness on the second day, each female was administered 50 µg/kg unlabelled BPA in 1 g peanut butter (n=5). At 1 h following administration of BPA, each female was anesthetized with isoflurane and perfused with 100 ml phosphate-buffered saline. Whole uteri were extracted, split at the midline, and each of two halves was placed in a separate pre-weighed 8 ml scintillation vial. Vials were immediately re-weighed to determine the wet mass of each sample and frozen at -20 °C.

2.5.2. Sample preparation, extraction, and derivatization

Samples were prepared following previously published procedures [64]. In brief, uterine samples were homogenized in 2 ml of acetate buffer solution (0.1 M, pH 5.0). One half of each uterus was treated with 2000 IU of β-glucuronidase to allow quantification of total BPA (aglycone plus conjugated forms of BPA), whereas the other half was untreated. All samples were thoroughly agitated and incubated at 37 °C for 18 h. After cooling, samples were spiked with 10 µl of propylsyringol-d_6 as recovery standard. Following previously published procedures [65], 6 ml of *n*-hexane was added to each sample. After shaking for 3 min, 2 ml of acetonitrile was added to the buffer solution. After shaking for an additional 3 min, the aqueous phase was extracted. Acrodisc syringe filters with GHP membrane (25 mm filtration area, 0.45 µm pore size, Pall Corporation,

Port Washington, NY) were conditioned with 5 ml acetonitrile prior to filtering the sample extracts.

Agilent Bond Elut Plexa SPE cartridges (60 mg bed mass, 3 ml volume, Agilent Technologies, Santa Clara, CA) were conditioned with 3 ml methanol and 3 ml water. Sample extracts were resuspended using 17 ml water and passed through SPE cartridges at a flow rate of 1-2 ml/min. Cartridges were then dried under vacuum for 10 min. BPA and recovery standards were eluted with 5 ml of dichloromethane and 50 µl of toluene at a flow rate of 1-2 ml/min. Eluents were then evaporated to 50 µl under a stream of nitrogen. Finally, 40 µl of MSTFA and 10 µl of pyrene-d_{10} were added as internal standard to the reaction vial prior to derivatization. Vials were then sealed and heated at 60 °C for 30 min. Following derivatization, 1 µl of the reaction mixture was injected into the GC-MS system.

2.5.3. Gas chromatography - mass spectrometry analysis

GC-MS analysis was conducted using a 6890 Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA) with a 5973 mass selective detector (MSD). The MSD was operated in full-scan mode from *m/z* 100 to 500 for qualitative determination and in selected ion-monitoring (SIM) mode for quantitative determination. Samples were analyzed following previously published procedures [65]. Quantification of BPA in uterine samples was calculated by comparison of peak intensity to 1 ng/µl BPA standard using ChemStation software.

2.5.4. Quality control for GC-MS analysis

Prior to sample analysis, these procedures were validated through recovery studies with a range of spiked uterine samples taken from rats not administered BPA. Recovery of BPA in uterine samples ranged from 71.7 to 83.5%, with a mean of 77.3%. The limit of detection was estimated at approximately 1 ng/g for uterine samples. Five blank vials were subjected to the same experimental procedure, except that they did not contain uterine tissue, to ensure that no BPA was introduced from external sources, such as reagents or laboratory equipment.

2.6. Experiment 4: Distribution of lower doses of ^{14}C-BPA in female mice

Isolated female mice were weighed and given a 1 g peanut butter supplement in a glass dish placed inside their cage on the day prior to the start of the experiment. At the onset of darkness on the first day, females were administered 0.5 µg/kg, 5 µg/kg, or 50 µg/kg ^{14}C-BPA in 0.2 g peanut butter (n=5 per condition; see Table S2). One additional contamination control animal was administered the equivalent dose of ethanol in peanut butter. At 1 h following administration of ^{14}C-BPA or ethanol, each female was anesthetized with isoflurane and blood collected via cardiac puncture. Animals were then perfused with 20 ml phosphate-buffered saline. Tissue samples were collected and placed in pre-weighed 8 ml scintillation vials. Tissues taken include a sample of the heart, lung, muscle from the hind leg, abdominal adipose, liver, a cross-section of the kidney encompassing both the cortex and medulla, both ovaries, and the whole uterus. All other procedures were identical to those of Experiment 1.

Table S2 Timing of treatments administered to female mice in Experiment 4. Ethanol and ^{14}C-BPA were given in a dietary supplement of peanut butter (PB).

	Day 1 8:00 AM	**Day 1 9:00AM**
Contamination Control	PB + ethanol	tissues sampled
0.5 µg/kg ^{14}C-BPA	PB + ^{14}C-BPA	tissues sampled
5 µg/kg ^{14}C-BPA	PB + ^{14}C-BPA	tissues sampled
50 µg/kg ^{14}C-BPA	PB + ^{14}C-BPA	tissues sampled

2.7. Experiment 5: Distribution of ^{14}C-BPA in female mice following repeated exposure

At the onset of light on the day prior to the start of the experiment, isolated female mice were weighed and randomly assigned to receive a single dose, 7 daily doses, or 28 daily doses of ^{14}C-BPA (n=5 per condition; see Table S3). For each condition, one additional contamination control animal was assigned to receive the equivalent dose of ethanol. At the onset of darkness on the first day, each female was administered 50 µg/kg ^{14}C-BPA in 0.2 g peanut butter. At the onset of darkness on subsequent days, females receiving multiple doses were each administered an additional dose of 50 µg/kg ^{14}C-BPA in 0.2 g peanut butter. At 24 h after administration of the final dose of ^{14}C-BPA, each female was anesthetized with isoflurane. All other procedures, including perfusion, blood and tissue collection, sample processing, and scintillation counting were identical to those of Experiment 4.

2.8. Statistical analyses

Data are presented as means ± SE. The threshold for statistical significance (α level) was set at $p < 0.05$. For planned comparisons between treatment conditions, data were subject to analysis of variance, and significant effects were followed by Newman-Keuls multiple comparisons. When equal doses of ^{14}C-BPA were administered, we focused statistical comparisons within each tissue across conditions. When doses of ^{14}C-BPA varied across treatment conditions, we conducted repeated measures analysis of variance across tissues.

Table S3 Timing of treatments administered to female mice in Experiment 5. Ethanol and ^{14}C-BPA were given in a dietary supplement of peanut butter (PB).

	Day 1 8:00 AM	**Day 2 8:00AM**	**Days 3-7 8:00AM**	**Day 8 8:00AM**	**Days 9-28 8:00AM**	**Day 29 8:00AM**
Single Dose	PB + ^{14}C-BPA	tissues sampled				
Contamination Control	PB + ethanol	tissues sampled				
7 Daily Doses	PB + ^{14}C-BPA	PB + ^{14}C-BPA	PB + ^{14}C-BPA	tissues sampled		
Contamination Control	PB + ethanol	PB + ethanol	PB + ethanol	tissues sampled		
28 Daily Doses	PB + ^{14}C-BPA	PB + ^{14}C-BPA	PB + ^{14}C-BPA	PB + ^{14}C-BPA	PB + ^{14}C-BPA	tissues sampled
Contamination Control	PB + ethanol	PB + ethanol	PB + ethanol	PB + ethanol	PB + ethanol	tissues sampled

3. Results

3.1. Experiment 1: Distribution and binding of ^{14}C-BPA in cycling female rats

Radioactivity from control animals not exposed to ^{14}C-BPA were at background levels (0.00 – 0.08 ng BPA/g), indicating no external contamination. For all peripheral tissues and serum (Fig. 1, Table 1), levels of radioactivity in animals treated with ^{14}C-BPA alone were in a completely non-overlapping range from these controls. Analysis of variance comparing the three conditions where animals were treated with ^{14}C-BPA showed a significant effect of condition in the uterus, $F(2,12) = 4.85$, $p = 0.028$, and serum, $F(2,12) = 9.98$, $p = 0.003$. Multiple comparisons indicated that significantly lower levels of radioactivity were observed in the uterus of animals given E_2 or ICI 182,780 pre-treatment, $p < 0.05$. Animals pre-treated with ICI 182,780 also showed significantly lower levels of radioactivity in serum compared to that of animals given ^{14}C-BPA alone, $p < 0.01$, and ^{14}C-BPA in combination with E_2, $p < 0.05$. Levels of radioactivity in all brain areas showed some overlap with control values and were much lower than those of peripheral tissues (Table 2).

Fig. 1 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lungs, muscle, adipose, uterus, and ovaries of cycling female rats following oral administration of 50 µg/kg ^{14}C-BPA, given alone or in combination with injections of either 17β-estradiol (E_2) or ICI 182,780 (Experiment 1). * differs from the ^{14}C-BPA condition, $p < 0.05$.

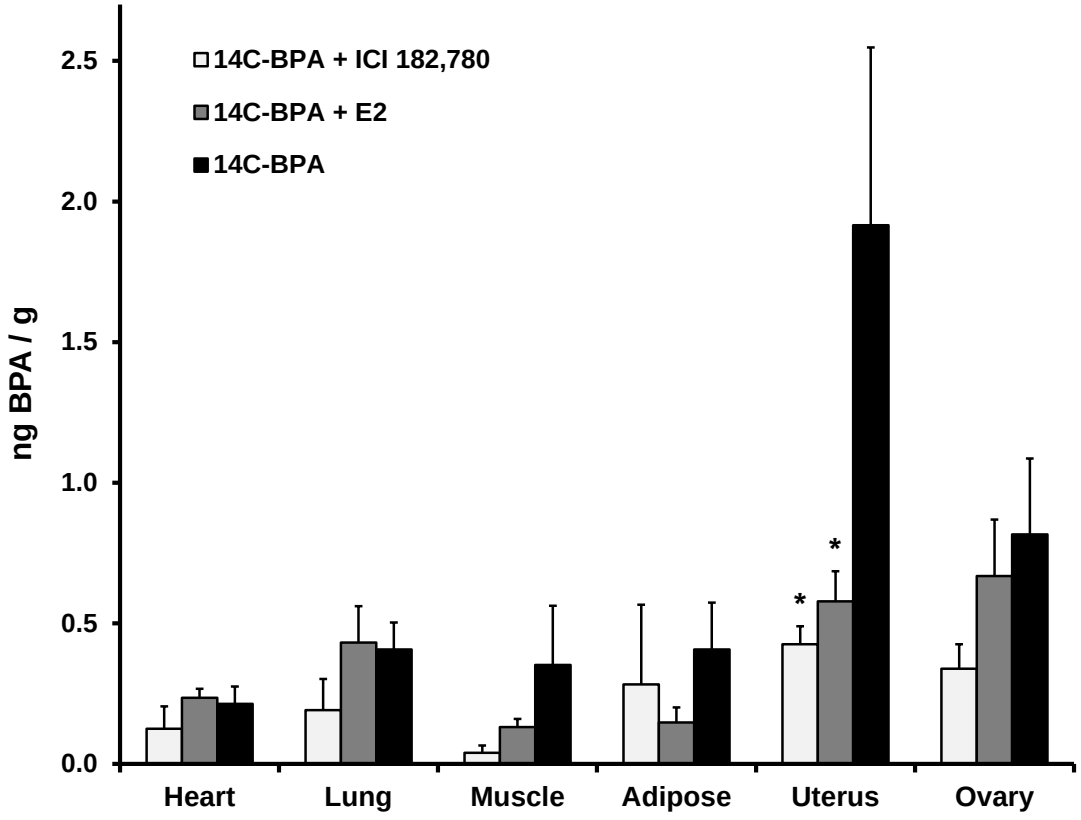


Table 1 Mean (±SE) radioactivity in serum (ng BPA/ml) and the liver and kidney (ng BPA/g) of cycling female rats (Experiment 1) and inseminated female rats (Experiment 2) following oral administration of 50 µg/kg ^{14}C-BPA, given alone or in combination with injections of either 17β-estradiol (E_2) or ICI 182,780. * differs from the ^{14}C-BPA condition, $p < 0.05$; ** differs from the ^{14}C-BPA condition, $p < 0.01$.

		^{14}C-BPA	**^{14}C-BPA + E2**	**^{14}C-BPA + ICI 182,780**
Expt. 1	**Serum**	12.03±1.93	8.54±2.49	0.78±0.27**
	Liver	9.21±0.94	6.53±1.19	5.33±1.16
	Kidney	1.42±0.14	1.43±0.25	1.45±0.25
Expt. 2	**Serum**	7.76±0.67	3.49±2.08*	1.13±0.41*
	Liver	8.53±1.34	5.64±2.50	8.82±2.20
	Kidney	2.02±0.22	1.21±0.43	1.40±0.09

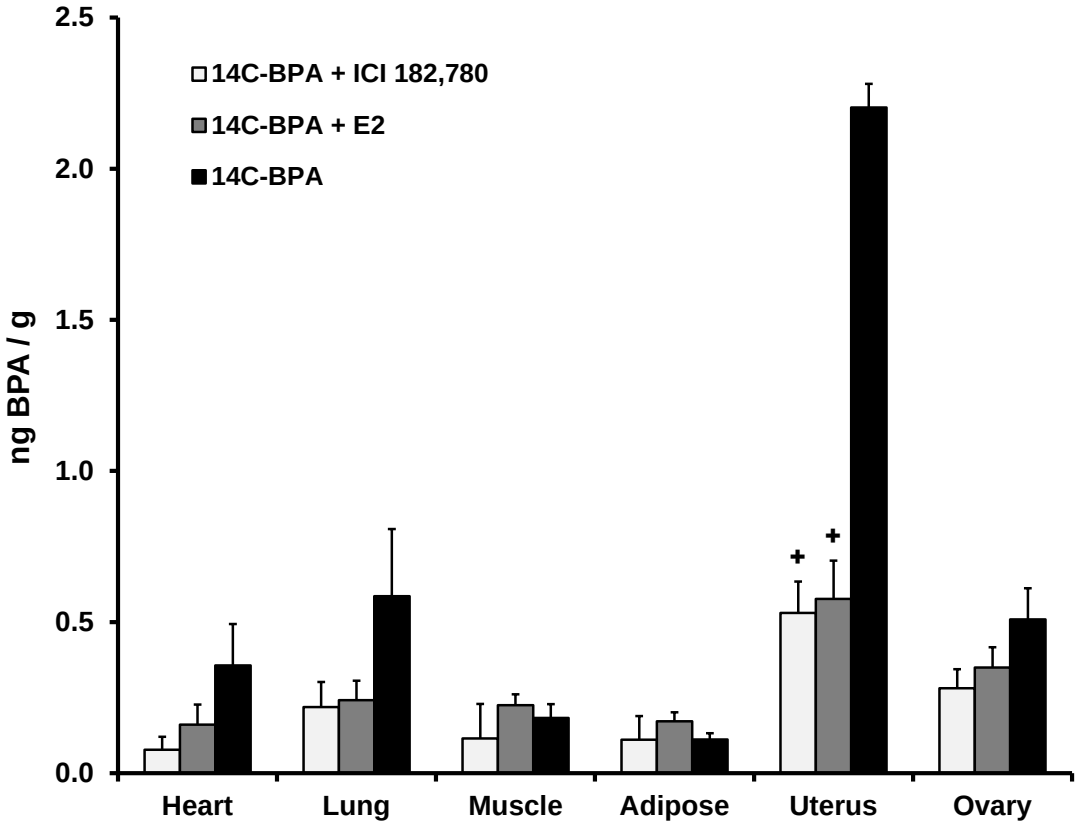
Table 2 Mean (±SE) radioactivity in the olfactory bulb, cerebellum, frontal cortex, striatum, hippocampus, hypothalamus, and amygdala (ng BPA/g) of cycling female rats (Experiment 1) and inseminated female rats (Experiment 2) following oral administration of 50 µg/kg ^{14}C-BPA.

	Experiment 1	**Experiment 2**
Olfactory Bulb	0.02±0.01	0.29±0.13
Cerebellum	0.03±0.01	0.11±0.04
Frontal Cortex	0.00±0.00	0.13±0.06
Striatum	0.01±0.01	0.17±0.08
Hippocampus	0.02±0.01	0.17±0.10
Hypothalamus	0.03±0.02	0.25±0.09
Amygdala	0.00±0.00	0.22±0.11

3.2. Experiment 2: Distribution and binding of ^{14}C-BPA in inseminated female rats

Radioactivity from control animals not exposed to ^{14}C-BPA were at background levels (0.00 - 0.07 ng BPA/g). For all peripheral tissues and serum (Fig. 2, Table 1), levels of radioactivity in animals treated with ^{14}C-BPA alone were in a completely non-overlapping range from these controls. Comparing the three conditions where animals were treated with ^{14}C-BPA showed significance in the uterus, $F(2,12) = 82.56$, $p < 0.001$, and serum, $F(2,12) = 6.88$, $p = 0.010$. Multiple comparisons indicated that significantly lower levels of radioactivity were observed in the uterus of animals given an E_2 or ICI 182,780 pre-treatment, $p < 0.001$. Animals pre-treated with E_2 or ICI 182,780 also showed significantly less radioactivity in serum compared to that of animals given ^{14}C-BPA alone, $p < 0.05$. Levels of radioactivity in all brain areas showed some overlap with control values (Table 2).

Fig. 2 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lungs, muscle, adipose, uterus, and ovaries of inseminated female rats following oral administration of 50 µg/kg ^{14}C-BPA, given alone or in combination with injections of either 17β-estradiol (E_2) or ICI 182,780 (Experiment 2). + differs from the ^{14}C-BPA condition, $p < 0.001$.



3.3. Experiment 3: Quantification of aglycone versus total BPA in the uterus of female rats

Table 3 shows the levels of aglycone and total (aglycone plus conjugated) BPA in the uterus of each of five rats. For aglycone and total BPA, the mean ± SE was 5.77 ± 0.45 ng/g and 8.07 ± 0.69 ng/g of uterine tissue, respectively. For all five rats, the majority of BPA present in the uterus was in aglycone form, with a range of 62.7 to 79.0 % and mean ± SE of 71.9 ± 3.4 % aglycone relative to total BPA. For all five blank samples, BPA levels were below the limit of detection.

Table 3 Aglycone and total BPA (ng/g) in uterine samples taken from cycling female rats (n=5) given 50 µg/kg BPA, as determined by GC-MS analysis (Experiment 3). Percent aglycone relative to total BPA is provided.

	Aglycone BPA (ng/g)	**Total BPA (ng/g)**	**% Aglycone**
Subject 1	4.18	6.36	65.7
Subject 2	5.70	7.22	79.0
Subject 3	5.81	7.36	79.0
Subject 4	6.92	9.46	73.2
Subject 5	6.24	9.96	62.7
Mean (±SE)	5.77±0.45	8.07±0.69	71.9±3.4

3.4. Experiment 4: Distribution of lower doses of ^{14}C-BPA in female mice

Radioactivity from control animals not exposed to ^{14}C-BPA were at background levels (0.00 ng BPA/g). Data for ^{14}C-BPA-treated animals are shown (Fig. 3, Table 4). Given the reliable pattern of radioactivity across peripheral tissues in both cycling and inseminated female rats, we hypothesized that, for each dose of ^{14}C-BPA, levels of radioactivity would be greater in the uterus of female mice than all other peripheral tissues not directly involved in conjugation or excretion of ingested ^{14}C-BPA. Excluding the liver, kidney, and serum from the analysis, there was a significant effect of tissue in the 50 µg/kg condition, $F(5,20) = 7.70$, $p < 0.001$; the 5 µg/kg condition, $F(5,20) = 7.65$, $p < 0.001$; and the 0.5 µg/kg condition, $F(5,20) = 4.17$, $p = 0.009$. Multiple comparisons indicated that elevated levels of radioactivity were present in the uterus compared to the heart, lung, muscle, adipose, and ovaries of animals administered 50 µg/kg ^{14}C-BPA, $p < 0.01$, as well as both 5 µg/kg and 0.5 µg/kg ^{14}C-BPA, $p < 0.05$. Levels of radioactivity in all brain areas showed some overlap with control values (Table 4).

Fig. 3 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lungs, muscle, adipose, uterus, and ovaries of female mice following oral administration of 50, 5, or 0.5 µg/kg ^{14}C-BPA (Experiment 4). * differs from all other tissues in the figure at the same dose, $p < 0.05$; ** differs from all other tissues in the figure at the same dose, $p < 0.01$.

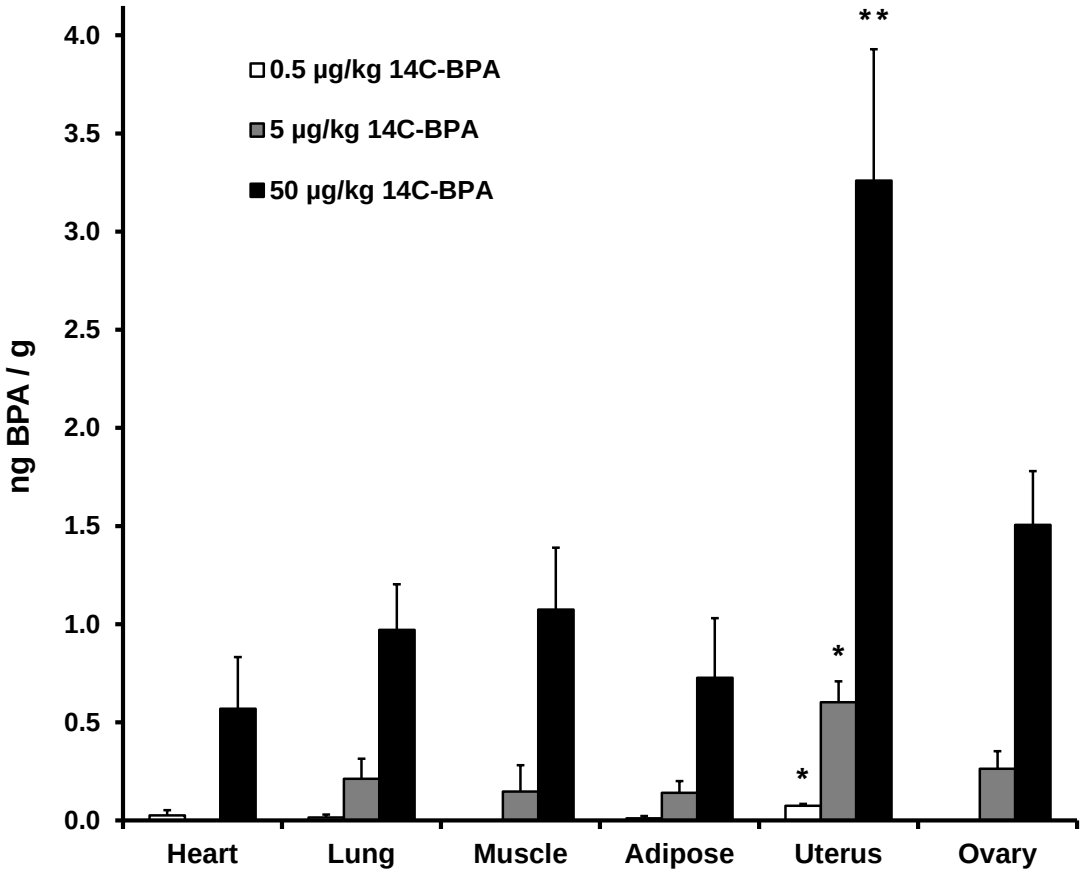


Table 4 Mean (±SE) radioactivity in serum (ng BPA/ml) and the liver, kidney, olfactory bulb, cerebellum, frontal cortex, and hypothalamus (ng BPA/g) of female mice following oral administration of 50, 5, or 0.5 µg/kg ^{14}C-BPA (Experiment 4). Given the complete absence of radioactivity in the brain at the 5 µg/kg dose, brain tissues were not analyzed for the 0.5 µg/kg dose.

	50 µg/kg	**5 µg/kg**	**0.5 µg/kg**
Serum	7.78±2.08	1.79±0.42	0.06±0.06
Liver	52.73±12.73	12.53±0.36	1.41±0.28
Kidney	18.13±5.21	5.80±0.77	0.80±0.09
Olfactory Bulb	0.03±0.02	0.00±0.00	N/A
Cerebellum	0.07±0.03	0.00±0.00	N/A
Frontal Cortex	0.01±0.01	0.00±0.00	N/A
Hypothalamus	0.03±0.03	0.00±0.00	N/A

3.5. Experiment 5: Repeated exposure to ^{14}C-BPA in female mice

Radioactivity from control animals not exposed to ^{14}C-BPA were at background levels (0.00 ng BPA/g), indicating no external contamination. Data for ^{14}C-BPA-treated animals are shown (Fig. 4, Table 5). Comparing only those animals treated with ^{14}C-BPA showed a significant effect of condition in the uterus, $F(2,12) = 5.75$, $p = 0.018$; ovaries, $F(2,12) = 4.27$, $p = 0.040$; liver, $F(2,12) = 26.68$, $p < 0.001$; and kidney, $F(2,12) = 28.64$, $p < 0.001$. In the uterus, multiple comparisons indicated that animals given 28 daily doses showed greater radioactivity than animals administered 7 daily doses or a single dose, $p < 0.05$. In the ovaries, higher levels of radioactivity were found in animals given 28 daily doses compared to animals administered a single dose, $p < 0.05$. For both the liver and kidney, higher levels of radioactivity were found in animals given 7 or 28 daily doses compared to those given a single dose, $p < 0.001$, while those given 28 daily doses showed greater radioactivity than those given 7 daily doses, $p < 0.05$. Levels of radioactivity in all brain areas showed some overlap with control values (Table 5).

Fig. 4 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lungs, muscle, adipose, uterus, and ovaries of female mice following oral administration of a single dose, 7 daily doses, or 28 daily doses of 50 µg/kg ^{14}C-BPA (Experiment 5). For all conditions, tissues were collected and analyzed for radioactivity 24 hours after the final dose of ^{14}C-BPA. * differs from the single dose condition, $p < 0.05$.

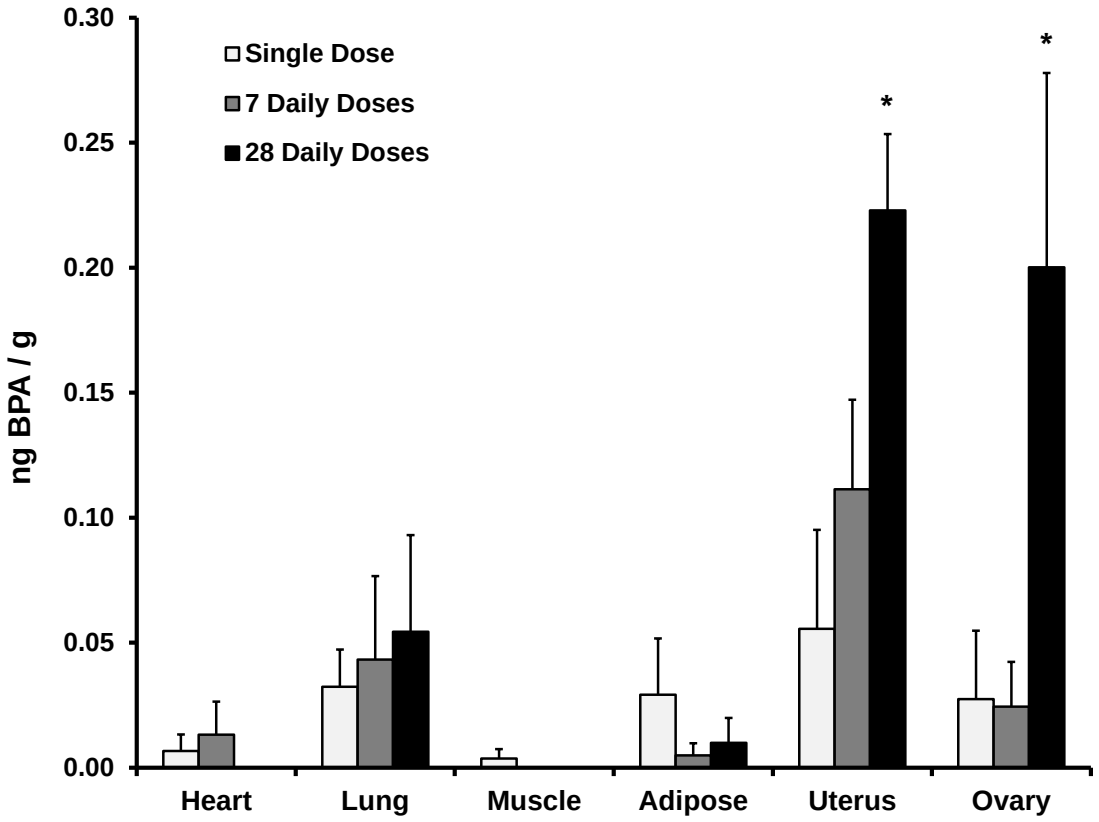


Table 5 Mean (±SE) radioactivity in serum (ng BPA/ml) and the liver, kidney, olfactory bulb, cerebellum, frontal cortex, and hypothalamus (ng BPA/g) of female mice following oral administration of a single dose, 7 daily doses, or 28 daily doses of 50 µg/kg ^{14}C-BPA (Experiment 5). For all conditions, tissues were collected and analyzed for radioactivity 24 hours after the final dose of ^{14}C-BPA. + differs from the single dose condition, $p < 0.001$.

	Single Dose	**7 Daily Doses**	**28 Daily Doses**
Serum	0.14±0.14	0.68±0.52	0.52±0.29
Liver	5.97±1.85	20.77±1.89+	27.60±2.60+
Kidney	3.58±0.78	7.84±0.44+	10.35±0.66+
Olfactory Bulb	0.00±0.00	0.00±0.00	0.00±0.00
Cerebellum	0.00±0.00	0.00±0.00	0.00±0.00
Frontal Cortex	0.00±0.00	0.00±0.00	0.00±0.00
Hypothalamus	0.00±0.00	0.00±0.00	0.02±0.02

4. Discussion

This research was designed to show distribution among tissues and bioavailability of BPA at doses lower than in previously published studies, after naturalistic dietary exposure. At these lower doses, levels in the uterus were substantially greater than observed in other tissues, apart from circulating BPA in serum and organs involved in metabolism (liver) and excretion (kidney). The doses used here are at or below the 50 µg/kg tolerable daily intake dose set by the United States Environmental Protection Agency [66] and the European Food Safety Authority [67]. According to Vandenberg *et al.* [52], a 50 µg/kg dose administered to rats is below the 400 µg/kg/day required to produce levels comparable to human blood concentrations. The bioavailability of BPA following oral consumption may be greater in humans than in rodents, given that humans do not possess keratinized buccal epithelium, thus permitting transmucosal absorption from the oral cavity [68].

When female rats received 50 µg/kg ^{14}C-BPA orally in Experiments 1 and 2, radioactivity was distributed throughout the body. Among previous studies using a single intragastric administration via gavage in rats, Snyder et al. [41] employed the much higher dose of 100 mg/kg ^{14}C-BPA, while Kim et al. [55] examined doses of 0.1 to 100 mg/kg. Kurebayashi et al. [56] examined metabolic clearance of doses as low as 20 µg/kg, but their lowest dose for tissue distribution was 100 µg/kg. Consistent with the findings of the present study, this previous work found that ^{14}C-BPA was distributed throughout the body and deposited in major organs, glands, and reproductive tissues of males and females [55,56]. The highest levels of ^{14}C-BPA were detected in the stomach

and intestines [41,55]; however, this finding is attributed to the metabolism and excretion of ingested BPA, and does not reflect deposition or binding to receptors in these tissues. Unlike these other studies which administered BPA via oral gavage, our administration involved the more naturalistic and non-invasive method of administration through a food supplement.

When females were treated with E_2 or the estrogen ICI 182,780 prior to ^{14}C-BPA administration in Experiments 1 and 2, less radioactivity was observed in the uterus and serum in comparison to females given ^{14}C-BPA alone. This effect was not significant in other tissues. Inseminated females (Experiment 2) showed a somewhat stronger effect than did cycling females (Experiment 1), likely due to greater homogeneity among inseminated females in hormonal condition and uterine ER, resulting in reduced variance among the females given ^{14}C-BPA alone. The especial presence of BPA observed in the uterus is consistent with the abundance of ERα and ERβ in that organ [69,70], and it also reflects the distribution of exogenous ^{3}H-E_2 observed following transdermal or nasal absorption [71,72]. Given high affinity of E_2 for ER [44,45], and potent actions of ICI 182,780 in blockade of ER [58,59], reduced radioactivity in the uterus of ^{14}C-BPA-treated females after pre-treatment with E_2 or ICI 182,780 is consistent with *in vivo* interaction of BPA with uterine nuclear (ERα, ERβ) and/or membrane (GPR30, mERα) ER. Nevertheless, binding factors in blood could also be involved given evidence in the current study of an impact of E_2 or ICI 182,780 upon radioactivity in serum. Similar strategies used in our laboratory have previously found that pre-treating females with E_2

can reduce uterine binding of ^{3}H-E_2 absorbed from cohabiting mice, despite potential metabolism in both individuals [73].

Further evidence that radioactivity observed in the uterus reflects bioactive, unconjugated BPA comes from the results of Experiment 3. As seen in Table 3, after female rats received 50 µg/kg, the majority of BPA at the uterus was determined to be aglycone (receptor-active) via GC-MS. We show that 71.9% of BPA in the uterus was aglycone at one hour after dietary ingestion of 50 µg/kg BPA. Some evidence suggests that β-glucuronidase activity intrinsic to tissues can result in unintended deconjugation during sample storage and processing; this has been reported for the placenta and fetal tissues [74], but its relevance to the uterus is not established. However, Doerge et al. [57], using LC/MS/MS and taking precautions to avoid unintended deconjugation, examined the relative distribution of aglycone and conjugated BPA at a number of organs. Their measures at two hours after i.v. administration of 100 µg/kg BPA indicated that 50% of BPA in the uterus was aglycone, whereas in the liver merely 2.5% was aglycone. Despite employing the same dose of BPA in Experiments 1, 2, and 3, we observed greater levels of total BPA in the uteri of rats in Experiment 3 compared to those of rats in Experiments 1 and 2. This finding could be due to different uteri sampling techniques used across these experiments. In Experiments 1 and 2, a smaller medial section between the vaginal canal and oviduct was taken to ensure complete solubilisation of the tissue, whereas the whole uterus was collected in Experiment 3. It is also possible that rats encountered environmental sources of BPA in addition to those of the experimental treatment, despite our precautions to ensure that dosing vehicles were uncontaminated. This would be very

unlikely to affect levels of radioactivity but could have had much more pronounced effects on uterine BPA concentrations as measured through GC-MS. Nevertheless, the data from Experiment 3 indicate that a substantial portion of the BPA localized to the uterus remains unconjugated.

In Experiment 4, we found that female mice given 50 µg/kg ^{14}C-BPA showed very similar results to those of female rats in Experiments 1 and 2. We also examined doses that were much lower than those examined in any previous study investigating the distribution of BPA. Mice given 0.5 or 5 µg/kg ^{14}C-BPA consistently showed greater levels of radioactivity in the uterus than in other non-metabolic organs. Among animals given 0.5 µg/kg ^{14}C-BPA, radioactivity was detected at levels above background in all subjects in the uterus, liver, and kidney, unlike other peripheral tissues where almost all animals showed undetectable levels. Radioactivity was also not detectable in brain tissues below the 50 µg/kg dose. Although serum BPA levels in mice given 50 µg/kg ^{14}C-BPA were higher than most reported human serum concentrations [1,11–13], the serum BPA levels in mice given 5 and 0.5 µg/kg ^{14}C-BPA were well within the range of measured human serum concentrations. To the best of our knowledge, these data are the first to show distribution of BPA among tissues at doses comparable to those in which humans are exposed.

When female mice in Experiment 5 received 1, 7, or 28 daily doses of 50 µg/kg ^{14}C-BPA, then measured 24 hours after the last dose, significantly more radioactivity was detected in the uterus, liver, and kidney following repeated doses. Previous studies [24,41,55–57] have only focused on the metabolism and distribution of single doses of

BPA, whereas repeated administration is more relevant to continuous human exposure. Some may have assumed that because complete clearance of BPA is evident within 24 to 48 hours after a single dose, the effects of BPA will not be exacerbated by repeated exposure. However, there was clearly a rise in radioactivity in the uterus after both 7 and 28 doses compared to the single dose. Furthermore, after 28 days of exposure, a significant effect emerged in the ovaries. Interestingly, serum BPA levels did not change following repeated doses, suggesting that elevations in tissue concentrations following continuous exposure are independent from blood concentrations. When measured 24 hours after the final dose of 50 µg/kg ^{14}C-BPA, serum BPA levels were within the range of reported human serum concentrations [1,11–13]. Some form of deposition or accumulation in tissues is possible, as is reduced metabolic clearance after repeated doses, consistent with the elevated levels in liver and kidney observed after repeated exposure. Similar elevations in tissue concentrations following multiple doses have been observed for other endocrine disruptors [75–77].

We have shown five lines of evidence that converge to indicate bioavailability of environmentally-relevant doses of dietary BPA in female reproductive organs. We consistently found that BPA is especially present in the uterus, where ER are abundant, and that the majority of uterine BPA likely exists in the aglycone form. Moreover, lower radioactivity in the uterus following concurrent exposure to E_2 or ICI 182,780 is concordant with BPA interaction with ER in this tissue. We also show for the first time that a single dose as low as 0.5 µg/kg reaches the uterus, and that daily exposure can

elevate the presence of BPA in the uterus and ovaries over levels induced by a single exposure.

The presence of BPA in female reproductive tissues does not necessarily imply the existence of toxicological effects. Nevertheless, given the potency of very low doses of natural E_2 [78–80], interference with its receptors and blood binding factors could set the stage for perturbed biological functioning. A variety of adverse reproductive effects of BPA have been observed in female laboratory rodents, including disruption of ova-implantation [62,63,81], abnormal uterine morphology [61], and altered reproductive tract development [82,83]. Although the doses for these effects greatly exceed doses examined in the current study, other findings such as abnormal meiotic cell division of oocytes in mice [84], advancement of pubertal development [85], and atypical maternal behaviour [86] are clearly within this range. Several adverse effects of BPA upon human reproductive health are known [87], however further research is clearly warranted.

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References

[1] Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reproductive Toxicology 2007;24:139–77.

[2] Al-Hiyasat AS, Darmani H, Elbetieha AM. Leached components from dental composites and their effects on fertility of female mice. European Journal of Oral Sciences 2004;112:267–72.

[3] Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenoestrogens released from lacquer coatings in food cans. Environmental Health Perspectives 1995;103:608–12.

[4] Biles JE, McNeal TP, Begley TH, Hollifield HC. Determination of bisphenol-A in reusable polycarbonate food-contact plastics and migration to food-simulating liquids. Journal of Agricultural and Food Chemistry 1997;45:3541–4.

[5] Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 1993;132:2279–86.

[6] Takao Y, Lee HC, Kohra S, Arizono K. Release of bisphenol A from food can lining upon heating. Journal of Health Science 2002;48:331–4.

[7] Crain DA, Eriksen M, Iguchi T, Jobling S, Laufer H, LeBlanc GA, et al. An ecological assessment of bisphenol-A: Evidence from comparative biology. Reproductive Toxicology 2007;24:225–39.

[8] Kang J-H, Kondo F, Katayama Y. Human exposure to bisphenol A. Toxicology 2006;226:79–89.

[9] Rodriguez-Mozaz S, López de Alda MJ, Barceló D. Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction–liquid chromatography–mass spectrometry. Journal of Chromatography A 2004;1045:85–92.

[10] Yamamoto T, Yasuhara A, Shiraishi H, Nakasugi O. Bisphenol A in hazardous waste landfill leachates. Chemosphere 2001;42:415–8.

[11] Teeguarden JG, Calafat AM, Ye X, Doerge DR, Churchwell MI, Gunawan R, Graham MK. Twenty-four hour human urine and serum profiles of bisphenol A during high-dietary exposure. Toxicological Sciences 2011;123:48–57.

[12] Sajiki J, Takahashi K, Yonekubo J. Sensitive method for the determination of bisphenol-A in serum using two systems of high-performance liquid chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 1999;736:255–61.

[13] Takeuchi T, Tsutsumi O. Serum bisphenol A concentrations showed gender differences, Possibly linked to androgen levels. Biochemical and Biophysical Research Communications 2002;291:76–8.

[14] Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environmental Health Perspectives 2005;113:391–5.

[15] Ye X, Kuklenyik Z, Needham LL, Calafat AM. Quantification of urinary conjugates of bisphenol A, 2,5-dichlorophenol, and 2-hydroxy-4-methoxybenzophenone in humans by online solid phase extraction–high performance liquid chromatography–

tandem mass spectrometry. Analytical and Bioanalytical Chemistry 2005;383:638–44.

[16] Liu Z, Wolff MS, Moline J. Analysis of environmental biomarkers in urine using an electrochemical detector. Journal of Chromatography B 2005;819:155–9.

[17] Calafat AM, Ye X, Wong L-Y, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-*tertiary*-octylphenol: 2003-2004. Environmental Health Perspectives 2008;116:39–44.

[18] Yang M, Kim S-Y, Chang S-S, Lee I-S, Kawamoto T. Urinary concentrations of bisphenol A in relation to biomarkers of sensitivity and effect and endocrine-related health effects. Environmental and Molecular Mutagenesis 2006;47:571–8.

[19] Chapin RE, Adams J, Boekelheide K, Gray LE, Hayward SW, Lees PSJ, et al. NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. Birth Defects Research Part B: Developmental and Reproductive Toxicology 2008;83:157–395.

[20] Geens T, Aerts D, Berthot C, Bourguignon JP, Goeyens L, Lecomte P, et al. A review of dietary and non-dietary exposure to bisphenol-A. Food and Chemical Toxicology 2012;50:3725–40.

[21] US Food and Drug Administration, 2013. Update on Bisphenol A (BPA): Use in Food Contact Application. http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm [accessed 12 April 2014]

[22] European Commission. Opinion of the scientific committee on food on bisphenol A. Brussels, Belgium: European Commission. 2002. Available: http://ec.europa.eu/food/fs/sc/scf/out128_en.pdf [accessed 20 November 2013]

[23] Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW. Pharmacokinetics of bisphenol A in neonatal and adult Sprague-Dawley rats. Toxicology and Applied Pharmacology 2010;247:158–65.

[24] Moors S, Diel P, Degen GH. Toxicokinetics of bisphenol A in pregnant DA/Han rats after single i.v. application. Archives of Toxicology 2006;80:647–55.

[25] Pottenger LH, Domoradzki JY, Markham DA, Hansen SC, Cagen SZ, Waechter JM. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicological Sciences 2000;54:3–18.

[26] Sieli PT, Jasarevic E, Warzak DA, Mao J, Ellersieck MR, Liao C, et al. Comparison of serum bisphenol A concentrations in mice exposed to bisphenol A through the diet versus oral bolus exposure. Environmental Health Perspectives 2011;119:1260–5.

[27] Taylor JA, Welshons WV, vom Saal FS. No effect of route of exposure (oral; subcutaneous injection) on plasma bisphenol A throughout 24 h after administration in neonatal female mice. Reproductive Toxicology 2008;25:169–76.

[28] Hanioka N, Naito T, Narimatsu S. Human UDP-glucuronosyltransferase isoforms involved in bisphenol A glucuronidation. Chemosphere 2008;74:33–6.

[29] Yokota H, Iwano H, Endo M, Koboyashi T, Inoue H, Ikushiro S, et al. Glucuronidation of the environmental oestrogen bisphenol A by an isoform of

UDP-glucuronosyltransferase, UGT2B1, in the rat liver. Biochem J 1999;340:405–9.

[30] Tukey RH, Strassburg CP. Human UDP-glucuronosyltransferases: Metabolism, expression, and disease. Annual Review of Pharmacology and Toxicology 2000;40:581–616.

[31] Völkel W, Colnot T, Csanády GA, Filser JG, Dekant W. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chemical Research in Toxicology 2002;15:1281–7.

[32] Upmeier A, Degen GH, Diel P, Michna H, Bolt HM. Toxicokinetics of bisphenol A in female DA/Han rats after a single i.v. and oral administration. Archives of Toxicology 2000;74:431–6.

[33] Doerge DR, Twaddle NC, Woodling KA, Fisher JW. Pharmacokinetics of bisphenol A in neonatal and adult rhesus monkeys. Toxicology and Applied Pharmacology 2010;248:1–11.

[34] Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, et al. Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. Molecular and Cellular Endocrinology 1998;142:203–14.

[35] Kuiper GG, Lemmen JG, Carlsson BO, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 1998;139:4252–63.

[36] Pennie WD, Aldridge TC, Brooks AN. Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. Journal of Endocrinology 1998;158:R11–R14.

[37] Dong S, Terasaka S, Kiyama R. Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. Environmental Pollution 2011;159:212–8.

[38] Wozniak AL, Bulayeva NN, Watson CS. Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-α–mediated Ca2+ fluxes and prolactin release in GH3/B6 pituitary tumor cells. Environmental Health Perspectives 2005;113:431–9.

[39] Matsushima A, Kakuta Y, Teramoto T, Koshiba T, Liu X, Okada H, et al. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERRγ. Journal of Biochemistry 2007;142:517–24.

[40] Okada H, Tokunaga T, Liu X, Takayanagi S, Matsushima A, Shimohigashi Y. Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-γ. Environmental Health Perspectives 2008;116:32–8.

[41] Snyder RW, Maness SC, Gaido KW, Welsch F, Sumner SCJ, Fennell TR. Metabolism and disposition of bisphenol A in female rats. Toxicology and Applied Pharmacology 2000;168:225–34.

[42] Matthews JB, Twomey K, Zacharewski TR. *In Vitro* and *in vivo* interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors α and β. Chemical Research in Toxicology 2001;14:149–57.

[43] Viñas R, Goldblum RM, Watson CS. Rapid estrogenic signaling activities of the modified (chlorinated, sulfonated, and glucuronidated) endocrine disruptor bisphenol A. Endocrine Disruptors 2013;1:e24511.

[44] Chun T, Gorski J. High concentrations of bisphenol A induce cell growth and prolactin secretion in an estrogen-responsive pituitary tumor cell line. Toxicology and Applied Pharmacology 2000;162:161–5.

[45] Kim HS, Han S-Y, Yoo SD, Lee BM, Park KL. Potential estrogenic effects of bisphenol-A estimated by *in vitro* and *in vivo* combination assays. The Journal of Toxological Sciences 2001;26:111–8.

[46] Welshons WV. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 2006;147:s56–s69.

[47] Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. *In vitro* molecular mechanisms of bisphenol A action. Reproductive Toxicology 2007;24:178–98.

[48] Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, et al. *In vivo* effects of bisphenol A in laboratory rodent studies. Reproductive Toxicology 2007;24:199–224.

[49] Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, et al. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicological Sciences 2002;68:121–46.

[50] Tyl RW, Myers CB, Marr MC, Sloan CS, Castillo NP, Veselica MM, et al. Two-generation reproductive toxicity study of dietary bisphenol A in CD-1 (Swiss) mice. Toxicological Sciences 2008;104:362–84.

[51] Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocrine Reviews 2009;30:75–95.

[52] Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee D-H, et al. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocrine Reviews 2012;33:378–455.

[53] Teeguarden JG, Hanson-Drury S. A systematic review of Bisphenol A "low dose" studies in the context of human exposure: A case for establishing standards for reporting "low-dose" effects of chemicals. Food and Chemical Toxicology 2013;62:935–48.

[54] Tanaka M, Kawamoto T, Matsumoto H. Distribution of 14C-bisphenol A in pregnant and newborn mice. Dental Materials 2010;26:e181–e187.

[55] Kim C, Sapienza P, Ross I, Johnson W, Luu H, Hutter J. Distribution of bisphenol A in the neuroendocrine organs of female rats. Toxicology and Industrial Health 2004;20:41–50.

[56] Kurebayashi H, Nagatsuka S-I, Nemoto H, Noguchi H, Ohno Y. Disposition of low doses of 14C-bisphenol A in male, female, pregnant, fetal, and neonatal rats. Archives of Toxicology 2005;79:243–52.

[57] Doerge DR, Twaddle NC, Vanlandingham M, Brown RP, Fisher JW. Distribution of bisphenol A into tissues of adult, neonatal, and fetal Sprague–Dawley rats. Toxicology and Applied Pharmacology 2011;255:261–70.

[58] Wade GN, Blaustein JD, Gray JM, Meredith JM. ICI 182,780: A pure antiestrogen that affects behaviors and energy balance in rats without acting in the brain. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 1993;265:R1392–R1398.

[59] Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Research 1991;51:3867–73.

[60] Chiu K, Lau WM, Lau HT, So K-F, Chang RC-C. Micro-dissection of rat brain for RNA or protein extraction from specific brain region. Journal of Visualized Experiments 2007;7:e269.

[61] Berger RG, Foster WG, deCatanzaro D. Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. Reproductive Toxicology 2010;30:393–400.

[62] Berger RG, Hancock T, deCatanzaro D. Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. Reproductive Toxicology 2007;23:138–44.

[63] Berger RG, Shaw J, deCatanzaro D. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. Reproductive Toxicology 2008;26:94–9.

[64] Zhang J, Cooke GM, Curran IHA, Goodyer CG, Cao X-L. GC–MS analysis of bisphenol A in human placental and fetal liver samples. Journal of Chromatography B 2011;879:209–14.

[65] Fernandez MF, Arrebola JP, Taoufiki J, Navalón A, Ballesteros O, Pulgar R, et al. Bisphenol-A and chlorinated derivatives in adipose tissue of women. Reproductive Toxicology 2007;24:259–64.

[66] Environmental Protection Agency. Integrated Risk Information System Substance File: Bisphenol A (CASRN 80-05-7). 1988. Available: http://www.epa.gov/iris/subst/0356.html [Accessed 20 November 2013]

[67] Aguilar F, Autrup H, Barlow S, Castle L, Crebelli R, Dekant W, et al. Toxicokinetics of bisphenol A scientific opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC). EFSA Journal 2008;759:1–10.

[68] Gayrard V, Lacroix MZ, Collet SH, Viguié C, Bousquet-Melou A, Toutain P-L, et al. High bioavailability of bisphenol A from sublingual exposure. Environmental Health Perspectives 2013;121:951–6.

[69] Kuiper GG, Carlsson BO, Grandien KAJ, Enmark E, Häggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997;138:863–70.

[70] Couse JF, Lindzey J, Grandien KAJ, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 1997;138:4613–21.

[71] Guzzo AC, Berger RG, deCatanzaro D. Excretion and binding of tritium-labelled oestradiol in mice (*Mus Musculus*): Implications for the Bruce effect. Reproduction 2010;139:255–63.

[72] Guzzo AC, Jheon J, Imtiaz F, deCatanzaro D. Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus Musculus*). Reproduction 2012;143:539–48.

[73] Guzzo AC, Pollock T, deCatanzaro D. Transfer of [3H]estradiol-17 and [3H]progesterone from conspecifics to cohabiting female mice. Journal of Endocrinology 2013;217:1–10.

[74] Waechter J Jr, Thornton C, Markham D, Domoradzki J. Factors affecting the accuracy of bisphenol A and bisphenol A-monoglucuronide estimates in mammalian tissues and urine samples. Toxicology Mechanisms and Methods 2007;17:13–24.

[75] Certa H, Fedtka N, Wiegand H-J, Müller AMF, Bolt HM. Toxicokinetics of *p*-tert-octylphenol in male Wistar rats. Archives of Toxicology 1996;71:112–22.

[76] Staskal DF, Diliberto JJ, Birnbaum LS. Impact of repeated exposure on the toxicokinetics of BDE 47 in mice. Toxicological Sciences 2006;89:380–5.

[77] Szabo DT, Diliberto JJ, Hakk H, Huwe JK, Birnbaum LS. Toxicokinetics of the flame retardant hexabromocyclododecane gamma: effect of dose, timing, route, repeated exposure, and metabolism. Toxicological Sciences 2010;117:282–93.

[78] deCatanzaro D, Baptista MA, Spironello-Vella E. Administration of minute quantities of 17β-estradiol on the nasal area terminates early pregnancy in inseminated female mice. Pharmacology Biochemistry and Behavior 2001;69:503–9.

[79] deCatanzaro D. Urinary oestradiol and testosterone levels from novel male mice approach values sufficient to disrupt early pregnancy in nearby inseminated females. Reproduction 2006;132:309–17.

[80] Ma W, Song H, Das SK, Paria BC, Dey SK. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Proceedings of the National Academy of Sciences 2003;100:2963–8.

[81] Crawford BR, deCatanzaro D. Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A. Reproductive Toxicology 2012;34:607–13.

[82] Kato H, Ota T, Furuhashi T, Ohta Y, Iguchi T. Changes in reproductive organs of female rats treated with bisphenol A during the neonatal period. Reproductive Toxicology 2003;17:283–8.

[83] Suzuki A, Sugihara A, Uchida K, Sato T, Ohta Y, Katsu Y, et al. Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reproductive Toxicology 2002;16:107–16.

[84] Hunt PA, Koehler KE, Susiarjo M, Hodges CA, Ilagan A, Voigt RC, et al. Bisphenol A exposure causes meiotic aneuploidy in the female mouse. Current Biology 2003;13:546–53.

[85] Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, Vom Saal FS. Environmental toxins: exposure to bisphenol A advances puberty. Nature 1999;401:763–4.

[86] Seta DD, Minder I, Dessì-Fulgheri F, Farabollini F. Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Research Bulletin 2005;65:255–60.

[87] Rochester JR. Bisphenol A and human health: a review of the literature. Reproductive Toxicology 2013;42:132–55.

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Chapter 3

Triclosan exacerbates the presence of ^{14}C-bisphenol A in tissues of female and male mice

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Authors' Contributions

Tyler Pollock: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

Brandon Tang: Assistance with data collection and data analysis.

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Abstract

Current human generations are commonly exposed to both triclosan (TCS), an antimicrobial agent, and bisphenol A (BPA), the monomer of polycarbonate plastics and epoxies. Both are readily absorbed into circulation and found distributed among diverse tissues. Potential interactions between TCS and BPA are largely unstudied. We investigated whether TCS exposure affects the distribution of ingested ^{14}C-BPA in select tissues. CF-1 mice were each subcutaneously injected with TCS then orally administered 50 µg/kg ^{14}C-BPA. Females received 0, 0.2, 0.6, 1, 2, or 18 mg TCS (equivalent respectively to 0, 6.3, 16.9, 30.1, 60.5, and 558.9 mg/kg). Males received 0, 0.2, 2, or 18 mg TCS (equivalent respectively to 0, 5.3, 53.4, and 415.0 mg/kg). Levels of radioactivity were measured through liquid scintillation counting in blood serum and brain, reproductive, and other tissues. Significantly elevated levels of radioactivity were observed following combined TCS and ^{14}C-BPA administration, with minimally effective TCS doses being tissue-dependent (Females: lungs, 0.6 mg; uterus, 1 mg; heart, muscle, ovaries, and serum, 18 mg. Males: serum, 0.2 mg; epididymides, 2 mg). Subsequently, we found that 2 or 6 mg TCS increased radioactivity in the ovaries and serum of females orally given only 5 µg/kg ^{14}C-BPA. These data indicate that TCS can interact with BPA *in vivo*, magnifying its presence in certain tissues and serum. The data are consistent with evidence that TCS utilizes enzymes that are critical for metabolism and excretion of BPA. Further research should investigate the mechanisms through which these two chemicals interact at environmentally-relevant doses.

Introduction

Triclosan (TCS) is an antimicrobial agent found in many household and consumer products, including soaps, dish sponges, cosmetics, skin cleansers, deodorants, toothpastes, and mouthwashes (Fang et al., 2010; Rodricks et al., 2010). Its bacteriostatic effects derive from interactions with enoyl-acyl carrier protein reductase enzymes, thereby inhibiting bacterial reproduction (Heath et al., 1999). This mechanism requires that free TCS be directly added to such consumer products, which incidentally permits rapid absorption of the chemical into the human body through the skin (Moss et al., 2000; Queckenberg et al., 2010) and gastrointestinal tract (Sandborgh-Englund et al., 2006). Although there are few published attempts to quantify human exposure, one study estimated that the average daily intake of TCS in North Americans is 350 µg (Nazaroff et al., 2012), which corresponds to 5 µg/kg for a 70 kg adult. TCS has been detected in human urine (Calafat et al., 2008a; Geens et al., 2009), serum (Allmyr et al., 2008; Geens et al., 2009), plasma (Allmyr et al., 2006), breast milk (Allmyr et al., 2006; Wang et al., 2011; Ye et al., 2008), as well as adipose and liver tissue (Geens et al., 2012).

Despite the fact that TCS exposure is ubiquitous, its capacity to act as an endocrine disruptor is not well studied. *In vitro* assays have shown that TCS binds to conventional estrogen receptor (ER) subtypes, ERα and ERβ, as well as the androgen receptor (Gee et al., 2008). When administered concurrently with their endogenous ligands, TCS inhibits the activity of these receptors (Gee et al., 2008; Henry and Fair, 2011), suggesting that TCS behaves in an antagonistic manner. However, *in vivo* uterotrophic assays have found that TCS itself elicits estrogenic effects (Jung et al.,

2012), and that TCS also potentiates the effects of ethinyl estradiol (Stoker et al., 2010). These estrogenic effects are supported by evidence that TCS inhibits estrogen sulfotransferases (James et al., 2010), thereby preventing metabolism of endogenous estrone and 17β-estradiol into their biologically inactive forms. TCS has also been shown to possess weak androgenic effects in fish (Foran et al., 2000) and anti-androgenic effects in rats (Kumar et al., 2009).

Bisphenol A (BPA) is a synthetic monomer used in the production of polycarbonate plastics and epoxy resins. BPA is found in numerous household and medical products, including drinking bottles, food storage containers, water pipes, and dental sealants (Vandenberg et al., 2007). BPA leaches out of these products into surrounding media through incomplete polymerization and depolymerization facilitated by exposure to heat (Biles et al., 1997; Krishnan et al., 1993). Ingestion of contaminated foods and beverages is the primary route of human exposure (Chapin et al., 2008; Kang et al., 2006; Vandenberg et al., 2007). Regulatory agencies have estimated adult daily intake of BPA from contaminated food sources at 0.1–0.2 µg/kg (US Food and Drug Administration, 2013) and 0.37 µg/kg (European Commission, 2002), which correspond to 7–14 µg and 25.9 µg for a 70 kg adult, respectively. Using data collected from the United States National Health and Nutrition Examination Survey (NHANES), estimates of adult daily BPA intake from urinary output measures are much lower, with 50^{th} percentiles ranging from 0.026–0.056 µg/kg and 95^{th} percentiles ranging from 0.151–0.289 µg/kg (Lakind and Naiman 2008, 2011; Lakind et al., 2012). These estimates correspond to ranges of 1.82–3.92 µg and 10.57–20.23 µg for a 70 kg adult, respectively.

The majority of ingested BPA is conjugated by hepatic sulfotransferases and UDP-glucuronosyltransferases (UGT) into several biologically inert forms (Hanioka et al., 2008; Nishiyama et al., 2002), with a smaller portion remaining unconjugated (Doerge et al., 2010). BPA has been detected in conjugated form in over 90% of adult urine samples (Calafat et al., 2005; Calafat et al., 2008b; Yang et al., 2006; Ye et al., 2005). BPA has also been measured in human serum (Sajiki et al., 1999; Teeguarden et al., 2011; Yoshimura et al., 2002), breast milk (Ye et al., 2006, 2008), placental tissue (Schönfelder et al., 2002), as well as adipose, brain, and liver tissue (Geens et al., 2012). However, these tissues are known to contain significant β-glucuronidase activity (Doerge et al., 2011; Moors et al., 2006); thus, potential deconjugation during sample storage and processing may have artificially increased measures of unconjugated BPA in those tissues.

BPA possesses estrogenic properties (Dodds and Lawson, 1936; Krishnan et al., 1993) and is capable of interacting with both ERα and ERβ (Gould et al., 1998; Kuiper et al., 1998). BPA also has high affinity for the membrane-bound estrogen receptor, GPR30 (Dong et al., 2011), and an orphan nuclear estrogen-related receptor, ERRγ (Matsushima et al., 2007). We (Pollock and deCatanzaro, submitted) have shown that ^{14}C-BPA, given orally to mice and rats at doses ranging from 0.5 to 50 µg/kg, distributes throughout the body and is especially present and bioavailable in the uterus where ER are abundant. Laboratory rodents exposed to BPA show altered embryonic and postnatal development (Takai et al., 2001; Tsutsui et al., 1998), pubertal development (Howdeshell et al., 1999), reproductive tract development (Kato et al., 2003; Suzuki et al., 2002), sexual

differentiation of the brain (Kubo et al., 2001, 2003), sexual behavior (deCatanzaro et al., 2013), fertility (Al-Hiyasat et al., 2002; Hunt et al., 2003), and maternal behavior (Palanza et al., 2002). The high levels of BPA exposure required to produce some of these effects, ranging from 1–1000 mg/kg (*e.g.* Kato et al., 2003; Kubo et al., 2001; Suzuki et al., 2002), has led to calls for further investigations into effects at lower, environmentally-relevant doses (Gray et al., 2004; vom Saal and Hughes, 2005). Some studies have shown estrogenic effects of BPA at doses below the EPA reference dose of 50 µg/kg, ranging from 0.05–30 µg/kg (*e.g.* Al-Hiyasat et al., 2002; deCatanzaro et al., 2013; Howdeshell et al., 1999; Hunt et al., 2003; Kubo et al., 2003; Palanza et al., 2002), whereas others have failed to find such evidence (Cagen et al., 1999; Ryan et al., 2010; Tyl et al., 2002).

Potential interactions between BPA and TCS are largely unstudied. Previous work in this laboratory has demonstrated that either TCS or BPA can disrupt blastocyst implantation in inseminated female mice (Berger et al., 2007, 2008, 2010; Crawford and deCatanzaro, 2012). Whereas a minimum of 6.75 mg BPA (Berger et al., 2010) or 18 mg TCS (Crawford et al., 2012) per day on days 1-5 of gestation was required to disrupt implantation, concurrent administration of 9 mg TCS and 2 mg BPA could do so, suggesting that these chemicals interact *in vivo* (Crawford and deCatanzaro, 2012). Consistent with these findings, TCS can inhibit BPA metabolism in human liver fractions (Wang et al., 2004). Here we provide evidence of *in vivo* interaction between BPA and TCS by measuring the distribution of ^{14}C-BPA administered in either the presence or absence of TCS throughout the bodies of adult mice. Compared to ^{14}C-BPA administered

alone, we showed elevated levels of radioactivity in blood serum and select tissues of male and female mice following administration of ^{14}C-BPA in the presence of TCS.

Materials and Methods

Animals and housing

Mice were housed in standard polypropylene cages measuring 28×16×11 cm with wire tops allowing *ad libitum* access to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. Females (32.7±2.7 g) and males (40.5±4.6 g) aged 3-4 months were of CF-1 strain and obtained from Charles River (Kingston, NY). The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. This research was approved by the Animal Research Ethics Board of McMaster University, conforming to the standards of the Canadian Council on Animal Care.

Chemicals and materials

TCS (5-chloro-2-[2,4-dichlorophenoxy]phenol, ≥97% purity) was obtained from Sigma-Aldrich, St. Louis, MO. ^{14}C-BPA ([ring-[^{14}C](U)]-BPA, in ethanol, 0.1 mCi/ml, 106 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, and 8 ml Midi-Vial scintillation vials were obtained from PerkinElmer, Waltham, MA.

Experimental design and dosing

At the onset of light on the first day of the experiment, animals were weighed, individually housed, and each given 1 g peanut butter in a glass dish placed inside their

cage. Regular rodent chow was withheld overnight to prevent dietary neophobia and ensure consumption of peanut butter. At the onset of darkness on the second day of the experiment, animals were randomly assigned to treatment conditions involving a single sc injection of TCS dissolved in 0.05 ml peanut oil. Females received 0, 0.2, 0.6, 1, 2, or 18 mg TCS (n=10 per dose) and males received 0, 0.2, 2, or 18 mg TCS (n=10 per dose). At 30 min after injection, each animal was orally administered 50 µg/kg ^{14}C-BPA in 0.2 g peanut butter. Water and bedding were removed prior to peanut butter administration to prevent contamination of the ^{14}C-BPA treatment. Table 1 provides TCS doses in mg/kg, and ^{14}C-BPA doses in µg and µCi for each treatment condition. At 1 h after administration of ^{14}C-BPA, each animal was anesthetized with isoflurane and blood was collected via cardiac puncture. Each animal was then perfused with 20 ml phosphate-buffered saline and tissues were collected and placed in pre-weighed scintillation vials. Tissue samples taken include the heart, lung, muscle from the hind leg, abdominal adipose, liver, and a cross-section of the kidney encompassing both the medulla and cortex. Female reproductive tissues collected include the whole uterus and both ovaries, whereas male reproductive tissues taken include one testis, one epididymis, and one vesicular gland (seminal vesicle). Specific areas of the brain were sampled only for animals that received 0 and 18 mg TCS (n=5 per dose). Brain areas collected include the olfactory bulbs, a caudal portion of the cerebellum, bilateral sections of the frontal cortex, and a portion of the hypothalamic region (posterior to the optic chiasm and anterior to the pituitary stalk on the ventral surface of the brain). Following tissue collection, vials were re-weighed to determine the wet mass of the sample.

Table 1 Mean (±SD) TCS doses in mg/kg and BPA doses in µg and µCi for each treatment condition.

	BPA Dose (µg/kg)	TCS Dose (mg)	TCS Dose (mg/kg)	BPA Dose (µg)	BPA Dose (µCi)
Females	50	0	0.0 ± 0.0	1.64 ± 0.16	0.76 ± 0.07
	50	0.2	6.3 ± 0.5	1.59 ± 0.13	0.74 ± 0.06
	50	0.6	16.9 ± 1.2	1.78 ± 0.13	0.83 ± 0.06
	50	1	30.1 ± 2.0	1.67 ± 0.11	0.78 ± 0.05
	50	2	60.5 ± 2.5	1.66 ± 0.07	0.77 ± 0.03
	50	18	558.9 ± 44.0	1.62 ± 0.14	0.75 ± 0.06
	5	0	0.0 ± 0.0	0.16 ± 0.01	0.08 ± 0.01
	5	2	66.2 ± 4.1	0.15 ± 0.01	0.07 ± 0.01
	5	6	184.6 ± 14.8	0.16 ± 0.01	0.08 ± 0.01
Males	50	0	0.0 ± 0.0	2.14 ± 0.13	0.99 ± 0.06
	50	0.2	5.3 ± 2.0	1.88 ± 0.09	0.87 ± 0.04
	50	2	53.4 ± 2.0	1.88 ± 0.07	0.87 ± 0.03
	50	18	415.0 ± 61.5	2.21 ± 0.31	1.03 ± 0.14

In a subsequent experiment, we administered a lower oral dose (5 µg/kg) of ^{14}C-BPA following sc injection of 0, 2, or 6 mg TCS. On the basis of stronger significance trends observed in females than males with combinations of TCS and the 50 µg/kg ^{14}C-BPA dose, this experiment was conducted only with females (n=10 per dose). Samples were not taken for brain tissues. In all other respects, procedures were identical to those described above.

Tissue processing for liquid scintillation counting

Following previously published procedures used in this laboratory (Guzzo et al., 2012, 2013; Pollock and deCatanzaro, submitted), tissue samples were solubilized by adding 1 ml SOLVABLE to each vial after re-weighing. Vials were then placed in a 50 °C water bath, agitated after 2 h, and left in the bath for an additional 2-3 h until completely dissolved. After removal from the bath and 10 min of cooling, 5 ml Ultima Gold scintillation cocktail was added to each vial. Vials were agitated again for 10 min to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyser (PerkinElmer) for 5 min to eliminate background noise in the form of heat and luminescence. Following this interval, radioactivity was measured for 5 min per vial, and final adjusted estimates for the amount of radioactivity per sample in disintegrations per minute (dpm) were automatically calculated by the accompanying Quanta-Smart software package. The dpm measure was standardized to the weight of the sample wet mass as dpm/g tissue, then converted to and reported as equivalent ng BPA/g tissue.

Serum processing for liquid scintillation counting

Following collection, blood samples were stored in uncoated microtubes and held in an upright position for at least 20 min to permit clotting. Following centrifugation at 1,500 *g* for 10 min, 10 µl serum was added to a scintillation vial containing 5 ml Ultima Gold. Vials containing serum were agitated for 10 min to promote mixing of the sample and scintillation cocktail. Measurement of radioactivity was performed as described above and reported as equivalent ng BPA/ml serum.

Contamination control procedures

Five females and five males were injected sc with 0.05 ml peanut oil and administered the equivalent dose of ethanol in 0.2 g peanut butter. All other details, including anesthesia and perfusion protocols, blood and tissue collection, sample processing, and scintillation counting were identical to those described above. Introduction of these animals into the experimental design was spread throughout the entire experiment to ensure that no external sources of radioactive contamination were present.

Statistical analyses

Data are presented as means + SE. The threshold for significance was set at $p<0.05$. Unless otherwise indicated, differences between treatments were analyzed by analysis of variance (ANOVA) for each tissue, with Bonferroni adjustments to correct for number of tissues. Significant ANOVA was followed by Newman-Keuls multiple comparisons between all pairs of treatment combinations. Bartlett's test showed equal

variances across treatments for females and males. We focused statistical comparisons on differences between treatments within each tissue rather than comparisons across tissues.

Results

Distribution of radioactivity in the presence of TCS in females given 50 µg/kg ^{14}C-BPA

Radioactivity was measured in the peripheral tissues of females who received a sc injection of TCS followed by oral administration of 50 µg/kg ^{14}C-BPA (Figs. 1 and 2). Comparisons were made between the six treatments for each of nine tissues, with the Bonferroni-corrected threshold for nine comparisons being $p<0.006$. ANOVA produced significant effects of treatment for the heart, $F(5,54)=8.24$, $p<0.001$; lung, $F(5,54)=6.16$, $p<0.001$; muscle, $F(5,54)=7.56$, $p<0.001$; uterus, $F(5,54)=7.20$, $p<0.001$; ovaries, $F(5,54)=3.70$, $p=0.005$; and serum, $F(5,54)=9.63$, $p<0.001$. Multiple comparisons revealed that the 18 mg TCS group differed from the 0 and 0.2 mg TCS groups across all tissues, and that the 18 mg TCS group also differed from the 0.6, 1, and 2 mg TCS groups for the heart, muscle, and serum. Furthermore, the 0.6 and 1 mg TCS groups differed from the 0 mg TCS group for the lung, and the 1 and 2 mg TCS groups differed from the 0, 0.2, and 0.6 mg TCS groups for the uterus.

Fig. 1 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lung, muscle, adipose, uterus, and ovaries of females following sc injection of 0, 0.2, 0.6, 1, 2, or 18 mg TCS and subsequent oral administration of 50 µg/kg ^{14}C-BPA. Difference from 0 mg TCS treatment in the same tissue: * $p < 0.05$; ** $p < 0.01$

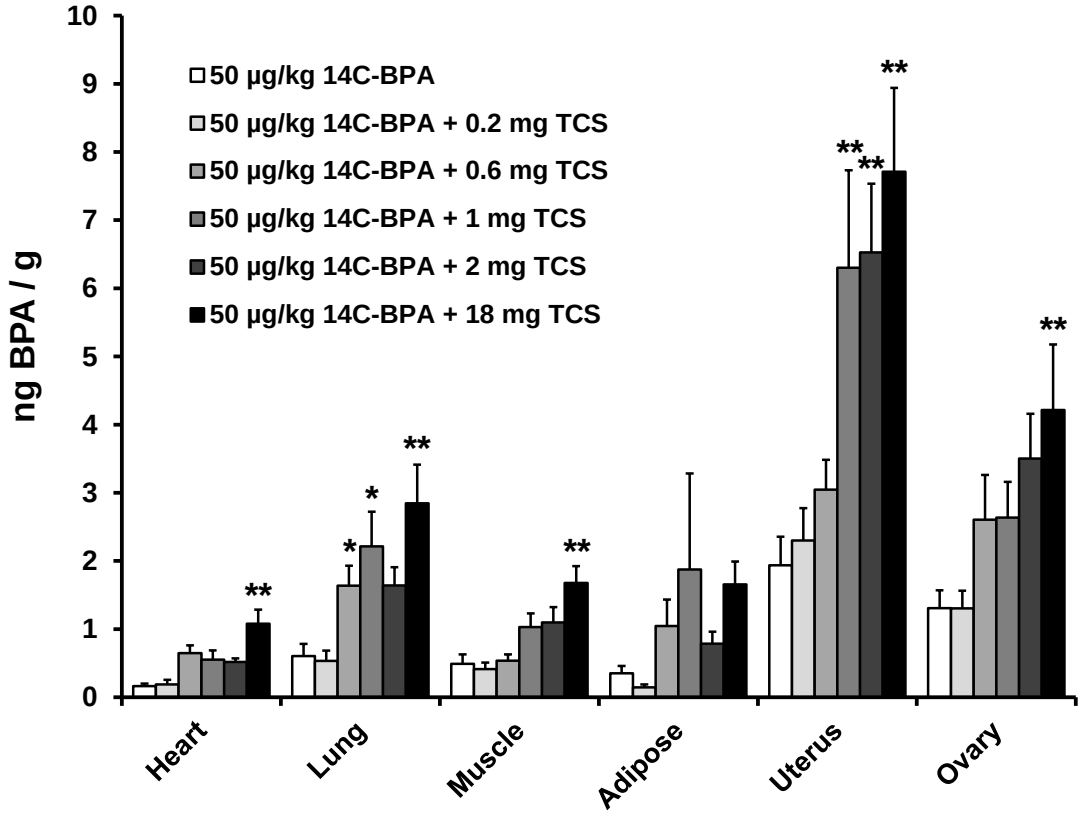
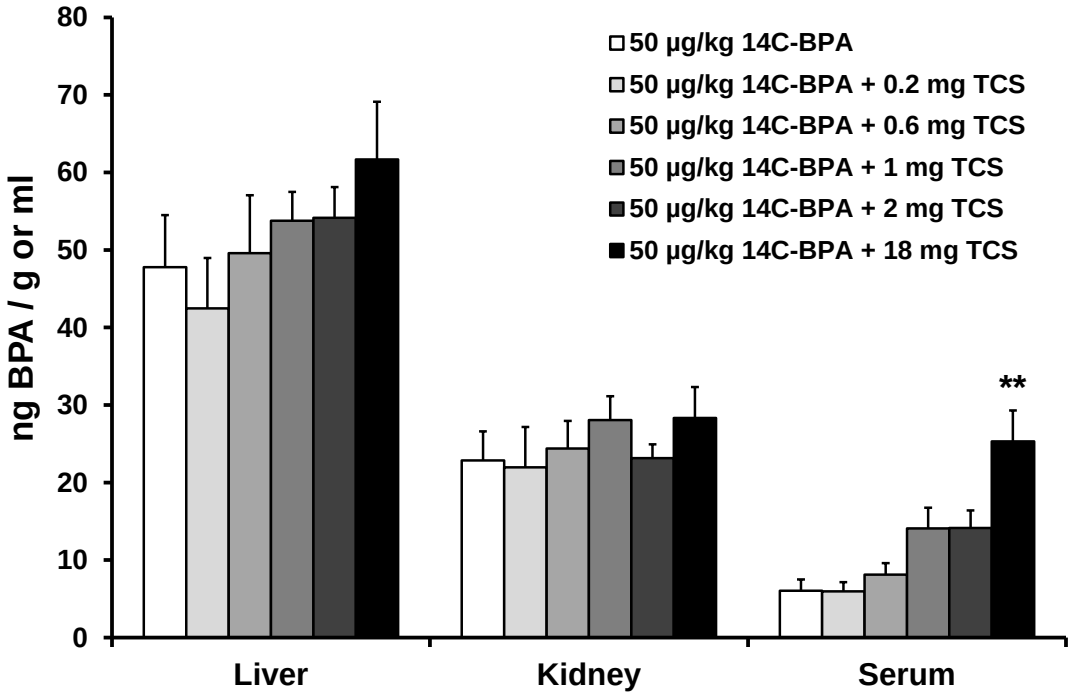


Fig. 2 Mean (+SE) concentration of radioactivity in the liver and kidney (ng BPA/g), as well as serum (ng BPA/ml) of females following sc injection of 0, 0.2, 0.6, 1, 2, or 18 mg TCS and subsequent oral administration of 50 µg/kg ^{14}C-BPA. Difference from 0 mg TCS treatment in the same tissue: ** $p < 0.01$



Distribution of radioactivity in the presence of TCS in males given 50 µg/kg ^{14}C-BPA

Radioactivity was measured in the peripheral tissues of males who received a sc injection of TCS followed by oral administration of 50 µg/kg ^{14}C-BPA (Figs. 3 and 4). Comparisons were made between the four treatments for each of ten tissues, with the Bonferroni-corrected threshold for ten comparisons being $p<0.005$. ANOVA yielded significant effects of treatment for the epididymides, $F(3,36)=9.67$, $p<0.001$; and serum, $F(3,36)=7.81$, $p<0.001$. Although non-significant after Bonferroni correction, the heart, $F(3,36)=3.10$, $p=0.039$; and lung, $F(3,36)=4.77$, $p=0.007$ approached significance. Multiple comparisons revealed that the 0.2, 2, and 18 mg TCS groups differed from the 0 mg TCS group for serum. Furthermore, the 2 and 18 mg TCS groups differed from the 0 mg TCS group for the epididymides.

Fig. 3 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lung, muscle, adipose, testes, epididymides, and seminal vesicles of males following sc injection of 0, 0.2, 2, or 18 mg TCS and subsequent oral administration of 50 µg/kg ^{14}C-BPA. Difference from 0 mg TCS treatment in the same tissue: ** $p < 0.01$

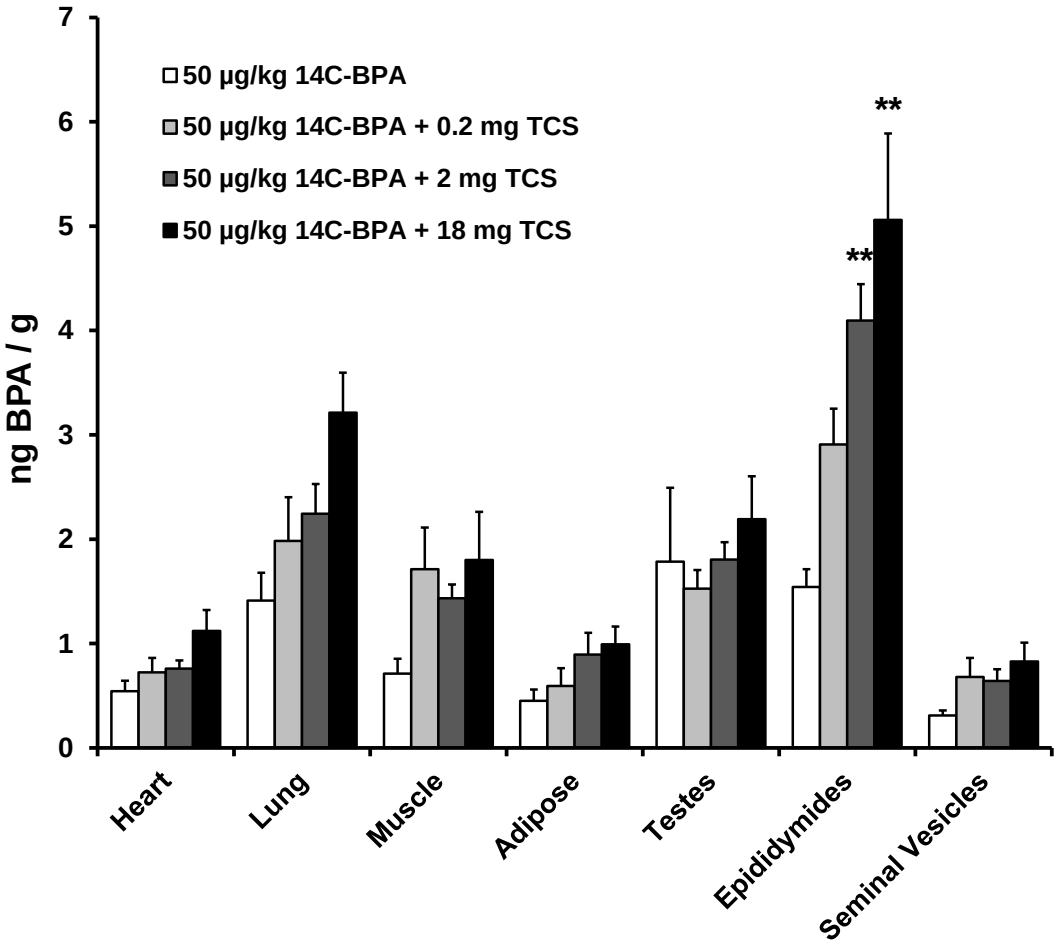
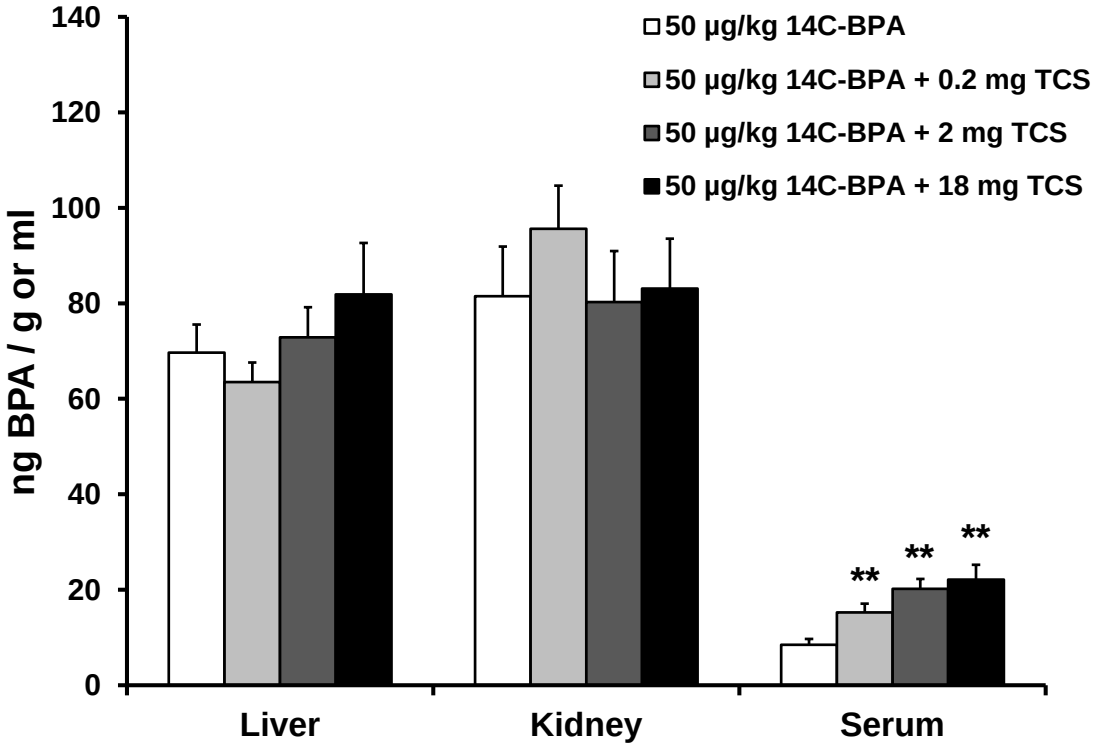


Fig. 4 Mean (+SE) concentration of radioactivity in the liver and kidney (ng BPA/g), as well as serum (ng BPA/ml) of males following sc injection of 0, 0.2, 2, or 18 mg TCS and subsequent oral administration of 50 µg/kg ^{14}C-BPA. Difference from 0 mg TCS treatment in the same tissue: * $p < 0.05$; ** $p < 0.01$



Distribution of radioactivity in the presence of TCS in the brain of females and males

Following sc injection of either 0 or 18 mg TCS and subsequent oral administration of 50 µg/kg ^{14}C-BPA, radioactivity was measured in select brain areas of female and male mice (Table 2). Uptake of radioactivity into the brain was much lower than that of peripheral tissues, and statistically significant differences among conditions were not observed.

Table 2 Mean (±SE) concentration of radioactivity, expressed as ng BPA/g, in the brain tissues of females and males following sc injection of either 0 or 18 mg TCS (n=5 per dose) and subsequent oral administration of 50 µg/kg ^{14}C-BPA. Statistically significant differences between treatment conditions were not observed.

	Olfactory Bulb	**Cerebellum**	**Frontal Cortex**	**Hypothalamus**
Female				
^{14}C-BPA	0.000 ± 0.000	0.029 ± 0.007	0.034 ± 0.018	0.000 ± 0.000
^{14}C-BPA + 18 mg TCS	0.036 ± 0.022	0.073 ± 0.007	0.020 ± 0.020	0.022 ± 0.022
Male				
^{14}C-BPA	0.118 ± 0.040	0.202 ± 0.055	0.098 ± 0.045	0.000 ± 0.000
^{14}C-BPA + 18 mg TCS	0.153 ± 0.094	0.265 ± 0.029	0.183 ± 0.029	0.000 ± 0.000

Distribution of radioactivity in the presence of TCS in females given 5 µg/kg ^{14}C-BPA

Radioactivity was evident in the peripheral tissues of females who received a sc injection of TCS followed by oral administration of 5 µg/kg ^{14}C-BPA (Figs. 5 and 6), but at substantially lower levels than were observed with females given 50 µg/kg ^{14}C-BPA described above. Trends toward increased radioactivity after either 2 or 6 mg TCS were seen in most tissues. Planned orthogonal *t*-tests comparing the 2 and 6 mg TCS groups with the 0 mg TCS group showed significance for the ovaries at both 2 mg TCS, $t(18)=2.37$, $p=0.014$, and 6 mg TCS, $t(18)=2.02$, $p=0.028$, and for serum at 2 mg TCS, $t(18)=1.77$, $p=0.045$.

Fig. 5 Mean (+SE) concentration of radioactivity, expressed as ng BPA/g, in the heart, lung, muscle, adipose, uterus, and ovaries of females following sc injection of 0, 2, or 6 mg TCS and subsequent oral administration of 5 µg/kg ^{14}C-BPA. Difference from 0 mg TCS treatment in the same tissue: * $p < 0.05$

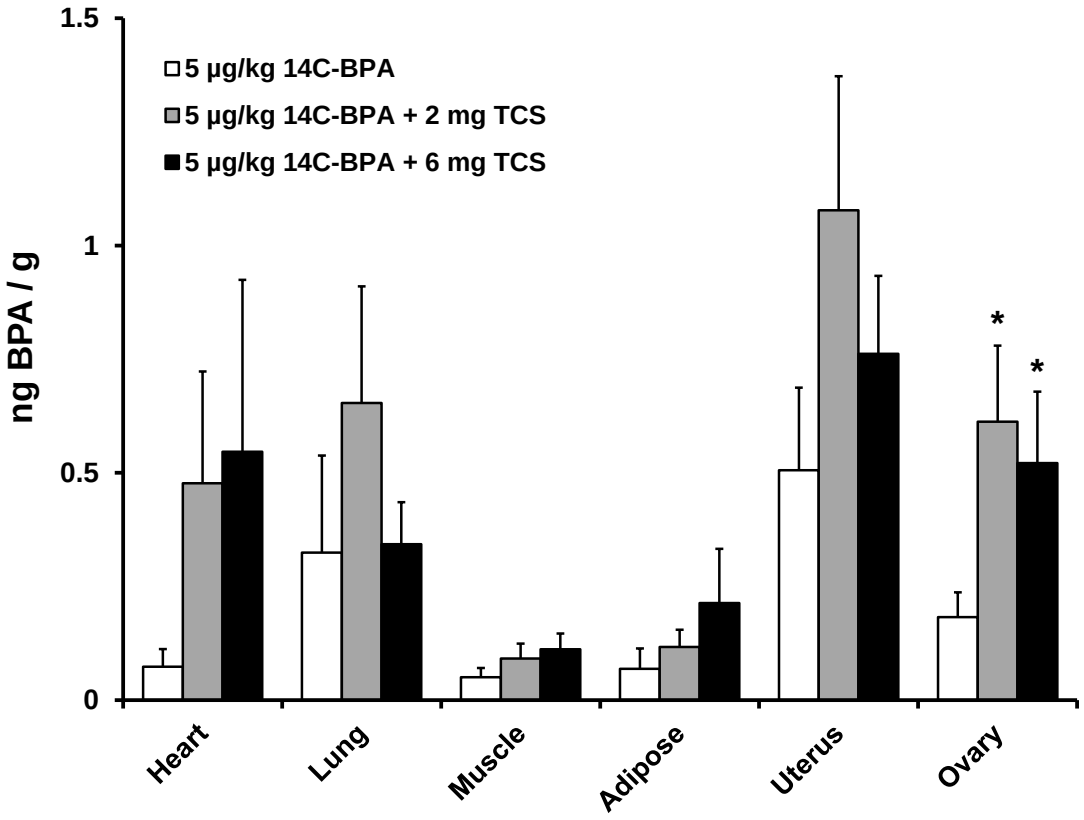
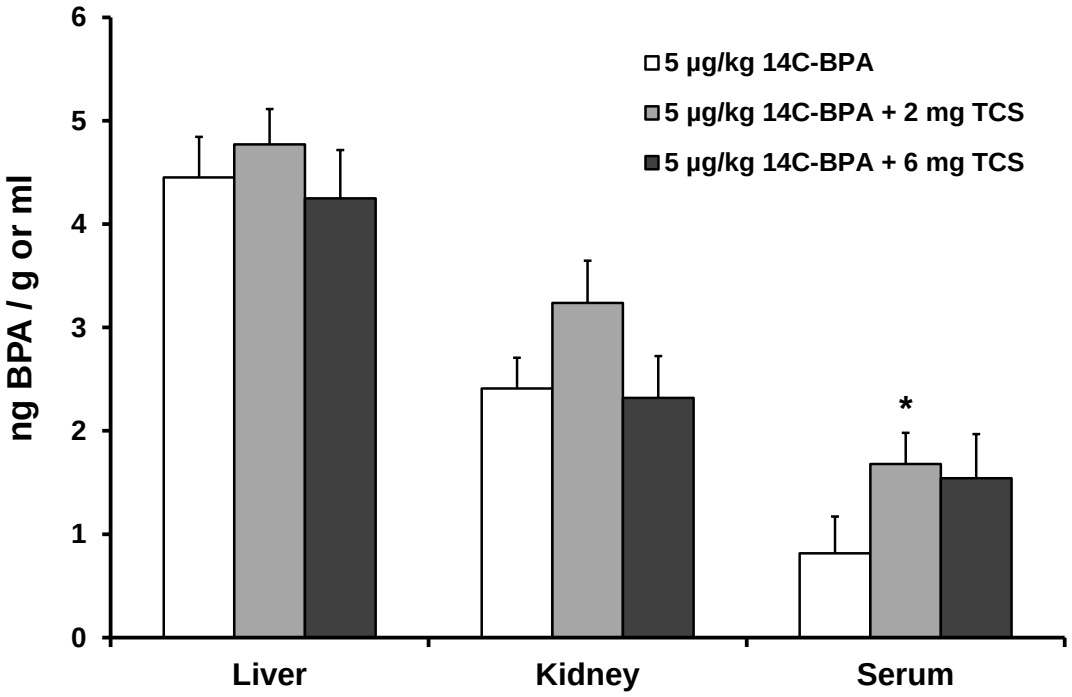


Fig. 6 Mean (+SE) concentration of radioactivity in the liver and kidney (ng BPA/g), as well as serum (ng BPA/ml) of females following sc injection of 0, 2, or 6 mg TCS and subsequent oral administration of 5 µg/kg ^{14}C-BPA. Difference from 0 mg TCS treatment in the same tissue: * $p < 0.05$



Levels of radioactivity in contamination control animals

Five females and five males were exposed to the same experimental design as treatment animals, except that these control animals received a non-radioactive peanut butter supplement. Measures of radioactivity for these animals were at baseline for serum (0 ng BPA/ml) and all tissues (0 ng BPA/g), except female adipose tissue (0.014 ng BPA/g) and female liver tissue (0.014 ng BPA/g). Concentrations of radioactivity in control animals were in a range entirely distinct from that of animals treated with ^{14}C-BPA, indicating no external sources of radioactive contamination.

Discussion

These data are the first to show that TCS potentiates the presence of ^{14}C-BPA in specific tissues of adult female and male mice. Levels of radioactivity were elevated in the heart, lungs, muscle, uterus, ovaries, and serum of females, as well as the epididymides and serum of males, when ^{14}C-BPA was administered in the presence of TCS compared to ^{14}C-BPA administered alone. We administered TCS via sc injection, as the prevalence of TCS in soaps likely leads to frequent percutaneous absorption (Perencevich et al., 2001), albeit with the caveat that percutaneous absorption is incomplete (Moss et al., 2000) and likely to be less efficient. We administered ^{14}C-BPA orally, given that ingestion of foods and beverages contaminated with BPA is the primary route of human exposure (Chapin et al., 2008; Kang et al., 2006; Vandenberg et al., 2007). We gave mice ^{14}C-BPA in peanut butter, as there is evidence that dietary BPA exposure results in less extensive first-pass hepatic elimination and higher levels of BPA

in serum compared to oral bolus administration (Sieli et al., 2011). The 30 min latency between triclosan and ^{14}C-BPA administration, and the 1 h latency between ^{14}C-BPA treatment and tissue collection, were chosen based on an effective paradigm used in previous studies involving the administration of ^{14}C-BPA in the presence of 17β-estradiol (Pollock and deCatanzaro, submitted).

The pattern of radioactivity across tissues observed in the present study is consistent with the differential expression of ER in these tissues. We observed the greatest impact of TCS administration on ^{14}C-BPA uptake, as evidenced by elevated levels of radioactivity, in the uterus, ovaries, and lungs of females, and in the epididymides of males. In females, the uterus and ovaries are characterized by high expression of ERα and ERβ (Couse et al., 1997; Kuiper et al., 1997). In males, the epididymides show moderate to high expression of both ER subtypes, whereas the testes express much lower levels (Couse et al., 1997). Aside from reproductive tissues, other peripheral tissues do not express significant levels of either ER subtype, except for ERα in the kidneys and ERβ in the lungs (Kuiper et al., 1997). Several brain areas possess cells that express ER, including the pituitary, hypothalamus, olfactory bulbs, and cerebral cortex (Couse et al., 1997); however, we observed much lower levels of radioactivity in the brain than in peripheral tissues. One study showed that, following administration of 100 µg/kg ^{14}C-BPA, approximately 95% of ^{14}C-BPA-derived radioactivity in plasma is bound to protein (Kurebayashi et al., 2003). Accordingly, low levels of radioactivity observed in brain tissues may indicate some difficulty of bound BPA in crossing the blood-brain barrier. Overall, our findings are consistent with previous work measuring

the distribution of radioactivity following oral administration of 50 µg/kg to 100 mg/kg ^{14}C-BPA alone (Kim et al., 2004; Pollock and deCatanzaro, submitted). We found through GC-MS that the majority of BPA reaching the uterus following oral administration of 50 µg/kg is unconjugated and bioavailable, and that both exogenous 17β-estradiol and the anti-estrogen ICI 182,780 compete with ^{14}C-BPA at the uterus (Pollock and deCatanzaro, submitted).

The observed exacerbation of ^{14}C-BPA uptake in the presence of TCS provides clear evidence of an *in vivo* interaction between BPA and TCS. We believe that these findings likely arise from inhibition of BPA conjugation by TCS actions at the enzymatic level. *In vitro*, TCS is capable of non-competitively inhibiting BPA sulfonation through sulfotransferase, as well as competitively inhibiting BPA glucuronidation through UGT (Wang et al., 2004). Moreover, a large portion of the free TCS present in the human body is localized within the liver (Geens et al., 2012). Taking these findings together, we suggest that TCS interacts with hepatic conjugating enzymes and inhibits the metabolism of ingested ^{14}C-BPA, thereby elevating levels of ^{14}C-BPA in circulation. This is concordant with our finding that ^{14}C-BPA administered in the presence of TCS resulted in significantly elevated radioactivity in serum.

Urinary measures from large samples of the U.S. population indicate widespread exposure to both BPA and TCS, with much variance among individuals in both cases (Calafat et al., 2008a, 2008b). Exposure to both substances is very common, but the degree of concurrent exposure is likely quite variable among individuals as there are different demographic correlates for the two substances. We observed that TCS clearly

enhanced *in vivo* presence of radioactivity in serum and tissues of mice after a dose of 50 µg/kg ^{14}C-BPA (the EPA reference dose), then replicated this with a dose of 5 µg/kg ^{14}C-BPA albeit with somewhat weaker effects. Whether these doses of BPA given to mice represent doses to which humans are exposed and whether they disrupt healthy endocrine functioning are matters of controversy, but a number of impacts of comparable doses of BPA have been reported in laboratory rodents (*cf.* Richter et al., 2007; Teeguarden and Hanson-Drury, 2013; Vandenberg et al., 2012). On the other hand, the doses of TCS that we employed are arguably much higher than those to which humans are commonly exposed. Relatively little is known about potential toxicity of TCS, even for high doses that might be associated with occupational exposure. We have only examined single acute doses and one temporal relationship between TCS and BPA exposure. Further investigations should explore the combined effects of TCS and BPA with both acute and chronic exposures at environmentally-relevant doses, with attention to mechanisms through which these chemicals can interact.

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References

Al-Hiyasat, A.S., Darmani, H., Elbetieha, A.M., 2002. Effects of bisphenol A on adult male mouse fertility. Eur. J. Oral Sci. 110, 163–167.

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. Sci. Total Environ. 372, 87–93.

Allmyr, M., Harden, F., Toms, L.L., Mueller, J.F., McLachlan, M.S., Adolfsson-Erici, M., Sandborgh-Englund, G., 2008. The influence of age and gender on triclosan concentrations in Australian human blood serum. Sci. Total Environ. 393, 162–167.

Berger, R.G., Foster, W.G., deCatanzaro, D., 2010. Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. Reprod. Toxicol. 30, 393–400.

Berger, R.G., Hancock, T., deCatanzaro, D., 2007. Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. Reprod. Toxicol. 23, 138–144.

Berger, R.G., Shaw, J., deCatanzaro, D., 2008. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. Reprod. Toxicol. 26, 94–99.

Biles, J.E., McNeal, T.P., Begley, T.H., Hollifield, H.C., 1997. Determination of bisphenol-A in reusable polycarbonate food-contact plastics and migration to food-simulating liquids. J. Agric. Food Chem. 45, 3541–3544.

Cagen, S.Z., Waechter, J.M., Dimond, S.S., Breslin, W.J., Butala, J.H., Jekat, F.W., Joiner, R.L., Shiotsuka, R.N., Veenstra, G.E., Harris, L.R., 1999. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol. Sci. 50, 36–44.

Calafat, A.M., Kuklenyik, Z., Reidy, J.A., Caudill, S.P., Ekong, J., Needham, L.L., 2005. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ. Health Perspect. 113, 391–395.

Calafat, A.M., Ye, X., Wong, L.Y., Reidy, J.A., Needham, L.L., 2008a. Urinary concentrations of triclosan in the U.S. population: 2003–2004. Environ. Health Perspect. 116, 303–307.

Calafat, A.M., Ye, X., Wong, L.Y., Reidy, J.A., Needham, L.L., 2008b. Exposure of the U.S. population to bisphenol A and 4-*tertiary*-octylphenol: 2003-2004. Environ. Health Perspect. 116, 39–44.

Chapin, R.E., Adams, J., Boekelheide, K., Gray, L.E., Hayward, S.W., Lees, P.S.J., McIntyre, B.S., Portier, K.M., Schnorr, T.M., Selevan, S.G., Vandenbergh, J.G., Woskie, S.R., 2008. NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. Birth Defects Res. B. Dev. Reprod. Toxicol. 83, 157–395.

Couse, J.F., Lindzey, J., Grandien, K.A.J., Gustafsson, J., Korach, K.S., 1997. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 138, 4613–4621.

Crawford, B.R., deCatanzaro, D., 2012. Disruption of blastocyst implantation by triclosan in mice: impacts of repeated and acute doses and combination with bisphenol-A. Reprod. Toxicol. 34, 607–613.

deCatanzaro, D., Berger, R.G., Guzzo, A.C., Thorpe, J.B., Khan, A., 2013. Perturbation of male sexual behavior in mice (*Mus musculus*) within a discrete range of perinatal bisphenol-A doses in the context of a high- or low-phytoestrogen diet. Food Chem. Toxicol. 55, 164–171.

Dodds, E.C., Lawson, W., 1936. Synthetic estrogenic agents without the phenanthrene nucleus. Nature 137, 996.

Doerge, D.R., Twaddle, N.C., Woodling, K.A., Fisher, J.W., 2010. Pharmacokinetics of bisphenol A in neonatal and adult rhesus monkeys. Toxicol. Appl. Pharmacol. 248, 1–11.

Doerge, D.R., Twaddle, N.C., Vanlandingham, M., Brown, R.P., Fisher, J.W., 2011. Distribution of bisphenol A into tissues of adult, neonatal, and fetal Sprague-Dawley rats. Toxicol. Appl. Pharmacol. 255, 261–270.

Dong, S., Terasaka, S., Kiyama, R., 2011. Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. Environ. Pollut. 159, 212–218.

European Commission, 2002. Opinion of the Scientific Committee on Food on bisphenol A. Brussels, Belgium: European Commission. Available: http://ec.europa.eu/food/fs/sc/scf/out128_en.pdf [accessed 29 November 2013].

Fang, J.L., Stingley, R.L., Beland, F.A., Harrouk, W., Lumpkins, D.L., Howard, P., 2010. Occurrence, efficacy, metabolism, and toxicity of triclosan. J. Environ. Sci. Heal. Part C 28, 147–171.

Foran, C.M., Bennett, E.R., Benson, W.H., 2000. Developmental evaluation of a potential non-steroidal estrogen: triclosan. Mar. Environ. Res. 50, 153–156.

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.

Geens, T., Neels, H., Covaci, A., 2009. Sensitive and selective method for the determination of bisphenol-A and triclosan in serum and urine as pentafluorobenzoate-derivatives using GC-ECNI/MS. J. Chromatogr. B. Biomed. Sci. App. 877, 4042–4046.

Geens, T., Neels, H., Covaci, A., 2012. Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. Chemosphere 87, 796–802.

Gould, J.C., Leonard, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D.P., Gaido, K.W., 1998. Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. Mol. Cell. Endocrinol. 142, 203–214.

Gray, G.M., Cohen, J.T., Cunha, G., Hughes, C., McConnell, E.E., Rhomberg, L., Sipes, I.G., Mattison, D., 2004. Weight of the evidence evaluation of low-dose reproductive and developmental effects of bisphenol A. Hum. Ecol. Risk Assess. Int. J. 10, 875–921.

Guzzo, A.C, Jheon, J, Imtiaz, F, deCatanzaro, D., 2012. Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). Reproduction 143, 539–548.

Guzzo, A.C., Pollock, T., deCatanzaro, D., 2013. Transfer of [3H]estradiol-17 and [3H]progesterone from conspecifics to cohabiting female mice. J. Endocrinol. 217, 1–10.

Hanioka, N., Naito, T., Narimatsu, S., 2008. Human UDP-glucuronosyltransferase isoforms involved in bisphenol A glucuronidation. Chemosphere 74, 33–36.

Heath, R.J., Rubin, J.R., Holland, D.R., Zhang, E., Snow, M.E., Rock, C.O., 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. J. Biol. Chem. 274, 11110–11114.

Henry, N.D., Fair, P.A., 2011. Comparison of *in vitro* cytotoxicity, estrogenicity and anti-estrogenicity of triclosan, perfluorooctane sulfonate and perfluorooctanoic acid: *in vitro* cytotoxicity and estrogenicity of emerging contaminants. J. Appl. Toxicol. 33, 265–272.

Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G., Vom Saal, F.S., 1999. Environmental toxins: exposure to bisphenol A advances puberty. Nature 401, 763–764.

Hunt, P.A., Koehler, K.E., Susiarjo, M., Hodges, C.A., Ilagan, A., Voigt, R.C., Thomas, S., Thomas, B.F., Hassold, T.J., 2003. Bisphenol A exposure causes meiotic aneuploidy in the female mouse. Curr. Biol. 13, 546–553.

James, M.O., Li, W., Summerlot, D.P., Rowland-Faux, L., Wood, C.E., 2010. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ. Int. 36, 942–949.

Jung, E.M., An, B.S., Choi, K.C., Jeung, E.B., 2012. Potential estrogenic activity of triclosan in the uterus of immature rats and rat pituitary GH3 cells. Toxicol. Lett. 208, 142–148.

Kang, J.H., Kondo, F., Katayama, Y., 2006. Human exposure to bisphenol A. Toxicology 226, 79–89.

Kato, H., Ota, T., Furuhashi, T., Ohta, Y., Iguchi, T., 2003. Changes in reproductive organs of female rats treated with bisphenol A during the neonatal period. Reprod. Toxicol. 17, 283–288.

Kim, C., Sapienza, P., Ross, I., Johnson, W., Luu, H., Hutter, J., 2004. Distribution of bisphenol A in the neuroendocrine organs of female rats. Toxicol. Ind. Health 20, 41–50.

Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., Feldman, D., 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132, 2279–2279.

Kubo, K., Arai, O., Ogata, R., Omura, M., Hori, T., Aou, S., 2001. Exposure to bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behavior in the rat. Neurosci. Lett. 304, 73–76.

Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R., Aou, S., 2003. Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res. 45, 345–356.

Kuiper, G.G., Carlsson, B.O., Grandien, K.A.J., Enmark, E., Häggblad, J., Nilsson, S., Gustafsson, J., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138, 863–870.

Kuiper, G.G., Lemmen, J.G., Carlsson, B.O., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139, 4252–4263.

Kumar, V., Chakraborty, A., Kural, M.R., Roy, P., 2009. Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with triclosan. Reprod. Toxicol. 27, 177–185.

Kurebayashi, H., Betsui, H., Ohno, Y., 2003. Disposition of a low dose of ^{14}C-bisphenol A in male rats and its main biliary excretion as BPA glucuronide. Toxicol. Sci. 73, 17–25.

Lakind, J.S., Naiman, D.Q., 2008. Bisphenol A (BPA) daily intakes in the United States: Estimates from the 2003-2004 NHANES urinary BPA data. J. Expo. Sci. Environ. Epidemiol. 18, 608–615.

Lakind, J.S., Naiman, D.Q., 2011. Daily intake of bisphenol A and potential sources of exposure: 2005-2006 National Health and Nutrition Examination Survey. J. Expo. Sci. Environ. Epidemiol. 21, 272–279.

Lakind, J.S., Levesque, J., Dumas, P., Bryan, S., Clarke, J., Naiman, D.Q., 2012. Comparing United States and Canadian population exposures from National Biomonitoring Surveys: Bisphenol A intake as a case study. J. Expo. Sci. Environ. Epidemiol. 22, 219–226.

Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M., Shimohigashi, Y., 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERRγ. J. Biochem. 142, 517–524.

Moors, S., Diel, P., Degen, G.H., 2006. Toxicokinetics of bisphenol A in pregnant DA/Han rats after single i.v. application. Arch. Toxicol. 80, 647–655.

Moss, T., Howes, D., Williams, F., 2000. Percutaneous penetration and dermal metabolism of triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether). Food Chem. Toxicol. 38, 361–370.

Nazaroff, W., Weschler, C.J., Little, J.C., Hubal, E.A.C., 2012. Intake to production ratio: a measure of exposure intimacy for manufactured chemicals. Environ. Health Perspect. 120, 1678–1683.

Nishiyama, T., Ogura, K., Nakano, H., Kaku, T., Takahashi, E., Ohkubo, Y., Sekine, K., Hiratsuka, A., Kadato, S., Watabe, T., 2002. Sulfation of environmental estrogens by cytosolic human sulfotransferases. Drug Metab. Pharmacokinet. 17, 221–228.

Palanza, P.L., Howdeshell, K.L., Parmigiani, S., vom Saal, F.S., 2002. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspect. 110, 415–422.

Perencevich, E.N., Wong, M.T., Harris, A.D., 2001. National and regional assessment of the antibacterial soap market: a step toward determining the impact of prevalent antibacterial soaps. Am. J. Infect. Control 29, 281–283.

Pollock, T., deCatanzaro, D., submitted. Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated oral administration at low doses. Reprod. Toxicol. MS#4140.

Queckenberg, C., Meins, J., Wachall, B., Doroshyenko, O., Tomalik-Scharte, D., Bastian, B., Abdel-Tawab, M., Fuhr, U., 2010. Absorption, pharmacokinetics, and safety of triclosan after dermal administration. Antimicrob. Agents Chemother. 54, 570–572.

Richter, C.A., Birnbaum, L.S., Farabollini, F., Newbold, R.R., Rubin, B.S., Talsness, C.E., Vandenbergh, J.G., Walser-Kuntz, D.R., vom Saal, F.S., 2007. *In vivo* effects of bisphenol A in laboratory rodent studies. Reprod. Toxicol. 24, 199-224.

Rodricks, J.V., Swenberg, J.A., Borzelleca, J.F., Maronpot, R.R., Shipp, A.M., 2010. Triclosan: a critical review of the experimental data and development of margins of safety for consumer products. Crit. Rev. Toxicol. 40, 422–484.

Ryan, B.C., Hotchkiss, A.K., Crofton, K.M., Gray, L.E., 2010. In utero and lactational exposure to bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility, and anatomy of female LE rats. Toxicol. Sci. 114, 133–148.

Sajiki, J., Takahashi, K., Yonekubo, J., 1999. Sensitive method for the detection of bisphenol-A in serum using two systems of high-performance liquid chromatography. J. Chromatogr. B. Biomed. Sci. App. 736, 255-261.

Sandborgh-Englund, G., Adolfsson-Erici, M., Odham, G., Ekstrand, J., 2006. Pharmacokinetics of triclosan following oral ingestion in humans. J. Toxicol. Environ. Health A 69, 1861–1873.

Schönfelder, G., Wittfoht, W., Hopp, H., Talsness, C.E., Paul, M., Chahoud, I., 2002. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ. Health Perspect. 110, A703–A707.

Sieli, P.T., Jasarevic, E., Warzak, D.A., Mao, J., Ellersieck, M.R., Liao, C., Kannan, K., Collet, S.H., Toutain, P.L., Vom Saal, F.S., Rosenfeld, C.S., 2011. Comparison of serum bisphenol A concentrations in mice exposed to bisphenol A through the diet versus oral bolus exposure. Environ. Health Perspect. 119, 1260-1265.

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

Suzuki, A., Sugihara, A., Uchida, K., Sato, T., Ohta, Y., Katsu, Y., Watanabe, H., Iguchi, T., 2002. Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reprod. Toxicol. 16, 107–116.

Takai, Y., Tsutsumi, O., Ikezuki, Y., Kamei, Y., Osuga, Y., Yano, T., Taketan, Y., 2001. Preimplantation exposure to bisphenol A advances postnatal development. Reprod. Toxicol. 15, 71–74.

Teeguarden, J.G., Calafat, A.M., Ye, X., Doerge, D.R., Churchwell, M.I., Gunawan, R., Graham, M.K., 2011. Twenty-four hour human urine and serum profiles of bisphenol A during high-dietary exposure. Toxicol. Sci. 123, 48–57.

Teeguarden, J.G., Hanson-Drury, S., 2013. A systematic review of Bisphenol A "low dose" studies in the context of human exposure: A case for establishing standards for reporting "low-dose" effects of chemicals. Food Chem. Toxicol. 62, 935–48.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Takahashi, M., Maizumi, N., Yamaguchi, F., Barrett, J.C., 1998. Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. Int. J. Cancer 75, 290–294.

Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J.C., 2002. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol. Sci. 68, 121–146.

US Food and Drug Administration, 2013. Update on Bisphenol A (BPA): Use in Food Contact Application. http://www.fda.gov/newsevents/publichealthfocus/ucm064437.htm [accessed April 2, 2014]

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.-H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V., 2007. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24, 139–177.

vom Saal, F.S., Hughes, C., 2005. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ. Health Perspect. 113, 926–933.

Wang, H., Zhang, J., Gao, F., Yang, Y., Duan, H., Wu, Y., Berset, J.D., Shao, B., 2011. Simultaneous analysis of synthetic musks and triclosan in human breast milk by gas chromatography tandem mass spectrometry. J. Chromatogr. B, 879, 1861–1869.

Wang, L., 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 Metab. Dispos. 32, 1162–1169.

Yang, M., Kim, S.Y., Chang, S.S., Lee, I.S., Kawamoto, T., 2006. Urinary concentrations of bisphenol A in relation to biomarkers of sensitivity and effect and endocrine-related health effects. Environ. Mol. Mutagen. 47, 571–578.

Ye, X., Bishop, A.M., Needham, L.L., Calafat, A.M., 2008. Automated on-line column-switching HPLC-MS/MS method with peak focusing for measuring parabens, triclosan, and other environmental phenols in human milk. Anal. Chim. Acta. 622, 150–156.

Ye, X., Kuklenyik, Z., Needham, L.L., Calafat, A.M., 2005. Quantification of urinary conjugates of bisphenol A, 2,5-dichlorophenol, and 2-hydroxy-4-methoxybenzophenone in humans by online solid phase extraction–high performance liquid chromatography–tandem mass spectrometry. Anal. Bioanal. Chem. 383, 638–644.

Ye, X., Kuklenyik, Z., Needham, L.L., Calafat, A.M., 2006. Measuring environmental phenols and chlorinated organic chemicals in breast milk using automated on-line column-switching-high performance liquid chromatography-isotope dilution tandem mass spectrometry. J. Chromatogr. B. Biomed. Sci. App. 831, 110–115.

Yoshimura, Y., Brock, J.W., Makino, T., Nakazawa, H., 2002. Measurement of bisphenol A in human serum by gas chromatography/mass spectrometry. Anal. Chim. Acta. 458, 331–336.

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Chapter 4

Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice

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Tyler Pollock: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

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Abstract

Triclosan, an antimicrobial agent added to personal care products, can modulate estrogenic actions. We investigated whether triclosan affects concentrations of exogenous and endogenous estradiol. Female mice were given injections of triclosan followed by 1 μCi tritium-labeled estradiol. Mice given daily 2-mg triclosan doses (57.9 mg/kg/dose) showed significantly elevated radioactivity in tissues and serum compared to controls. A single dose of 1 or 2 mg triclosan increased radioactivity in the uterus in both cycling and peri-implantation females. We also measured natural urinary estradiol at 2–12 h following triclosan injection. Unconjugated estradiol was significantly elevated for several hours following 1 or 2 mg of triclosan. These data are consistent with evidence that triclosan inhibits sulfonation of estrogens by interacting with sulfotransferases, preventing metabolism of these steroids into biologically inactive forms. Elevation of estrogen concentrations by triclosan is potentially relevant to anti-reproductive and carcinogenic actions of excessive estrogen activity.

1. Introduction

Triclosan (CAS 3380-34-5) is a synthetic biocide designed to inhibit bacterial reproduction by interacting with enoyl-acyl carrier protein reductase enzymes [1]. It is added to many consumer and household products, including soaps, dish sponges, cosmetics, deodorants, toothpastes, mouthwashes, clothing, and children's toys [2–4]. Dermal contact with these products leads to rapid absorption of triclosan into the body through the skin [5,6], while oral ingestion leads to uptake through the gastrointestinal tract [7]. Based on the 2003–2004 U.S. National Health and Nutrition Examination Survey (NHANES), 74.6% of the 2,517 human urine samples contained detectable levels of triclosan, with concentrations ranging from 2.4 to 3,790 µg/L [8]. Detection frequency of urinary triclosan in the U.S. population reached a peak between 2007–2008 at 80.8%, but has since fallen to 72.0% as of 2011–2012 [9]. Similarly, mean urinary triclosan concentrations in the U.S. population peaked in 2005–2006 at 18.8 µg/L but fell to 12.46 µg/L as of 2011–2012 [9]. Triclosan has also been detected in human serum [10,11], plasma [12], breast milk [12,13], and adipose and liver tissue [14].

Triclosan has known estrogenic effects, including stimulating breast and ovarian cancer cell growth *in vitro* [15,16] and magnifying the effects of ethinyl estradiol in rodent uterotrophic assays [17,18]. However, the mechanisms underlying these effects are not well understood. Triclosan binds to both conventional estrogen receptor (ER) subtypes, ERα and ERβ [19,20]. Thus, exposure to triclosan may induce estrogenic effects by directly activating ER. Triclosan also potently inhibits hepatic sulfotransferase activity [21–23], thereby reducing sulfonation of endogenous estrogens such as E_2 and

xenoestrogens such as bisphenol A (BPA) [23]. Thus, exposure to triclosan may potentiate *in vivo* estrogenic effects by preventing metabolism of estrogens to their biologically inactive forms.

Previous work in this laboratory demonstrated *in vivo* interactions between triclosan and BPA. When mice were given a single dose of triclosan ranging from 0.2–18 mg, greater levels of ^{14}C-BPA were detected in serum and tissues including the heart, lung, muscle, uterus, ovaries, and epididymides, than in animals given ^{14}C-BPA alone [24]. Other studies indicated that either triclosan or BPA can disrupt blastocyst implantation in inseminated female mice [25–29], and that doses of BPA or triclosan that were insufficient on their own to have effects could disrupt implantation when the two substances were given concurrently [29]. These findings are consistent with the notion that triclosan inhibits BPA conjugation [23], permitting higher levels of BPA to interact with ER in tissues such as the uterus.

Whereas BPA is a weakly estrogenic environmental chemical, E_2 is the most potent natural estrogen. Any deviations from normal E_2 levels might lead to adverse health effects, as estrogen levels are tightly regulated and play critical roles in development, fertility, and behavior [30]. Of especial importance to human health is the consistent finding that elevated E_2, often through hormone-replacement therapy, is associated with an increased risk of breast [31], endometrial [32], and ovarian [33] cancers. Also, in inseminated females, minute elevations in estrogen activity can impede blastocyst implantation, leading to pregnancy failure [30,34]. Given the fact that triclosan exposure is ubiquitous, its potential capacity to modulate estrogen levels or activity *in*

vivo is worthy of investigation. Here we investigated the impact of single or repeated triclosan injections on concentrations of exogenous tritium-labeled estradiol (^{3}H-E_2) and endogenous urinary E_2. We hypothesized that a single injection of triclosan would elevate ^{3}H-E_2 levels in reproductive tissues of cycling and peri-implantation female mice, and that this effect would be more pronounced with repeated triclosan injections over multiple days. We also hypothesized that triclosan administration would increase endogenous E_2 concentrations as measured in urine.

2. Materials and Methods

2.1. Animals and housing

Female mice aged 3–5 months were of CF–1 strain and obtained from Charles River (Kingston, NY). Animals were housed in standard polypropylene cages measuring 28×16×11 (l×w×h) cm with wire tops allowing *ad libitum* access to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University.

2.2. Chemicals and materials

Triclosan (CAS 3380-34-5, 5-chloro-2-[2,4-dichlorophenoxy]phenol, ≥97% purity) was obtained from Sigma-Aldrich, St. Louis, MO. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, 8 ml midi-vial scintillation vials, and [2,4,6,7-[^{3}H](N)]-E_2 (stock solution in ethanol, 1.0 µCi/µl, 81.0 Ci/mmol) were obtained

from PerkinElmer, Waltham, MA. E_2 and creatinine standards were obtained from Sigma-Aldrich, Oakville, ON, Canada. E_2 antibodies and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA.

2.3. Experiment 1: Measurement of ^{3}H-E_2 in cycling females after repeated triclosan doses

At the onset of darkness on the first day of the experiment, 21 cycling female mice with regular estrous cycles were weighed (39.4 ± 5.6 g), individually housed, and each given a subcutaneous (sc) injection of 0, 1, or 2 mg triclosan (corresponding to 0.0 ± 0.0, 23.2 ± 2.5, or 57.9 ± 3.9 mg triclosan/kg bodyweight, respectively) dissolved in 0.05 ml peanut oil (n=7 per dose). These injections were repeated at the same time on days 2 through 7, such that each mouse had a total of 7 injections of the same dose. We rotated injection locations of triclosan among the neck, right flank, and left flank to prevent irritation of the injection site. At 24 h after the final triclosan dose, each animal was given an intraperitoneal (ip) injection of 1 µCi ^{3}H-E_2 (corresponding to 3.36 ng E_2) in 9 µl phosphate-buffered saline (PBS). At 1 h after ^{3}H-E_2 administration, each animal was anesthetized with isoflurane and blood was collected via cardiac puncture. Each animal was perfused with 15 ml PBS and tissues were collected in pre-weighed scintillation vials. Tissue samples taken include the heart, lung, liver, superficial adductor muscle from the hind leg, the whole uterus, both ovaries, and a cross-section of the kidney encompassing both the medulla and cortex. Vials were re-weighed following tissue collection to determine the sample wet mass.

We administered triclosan via sc injection in all experiments, as the presence of triclosan in personal care products can lead to transdermal absorption. Percutaneous penetration is incomplete compared to sc injection; one estimate suggests that transdermal absorption of triclosan is 6.3 ± 1.1 % in humans and 22.8 ± 4.6 % in rats within 24 h [5]. The 1 and 2 mg triclosan doses were chosen based on the lowest effective doses in a previous study showing an interaction between triclosan and ^{14}C-BPA [24]. ^{3}H-E_2 was given ip to facilitate systemic distribution of the steroid [35], and the 1 µCi dose of ^{3}H-E_2 represents just a small fraction of the animals' endogenous E_2 [35,36]. Finally, the 24-h latency between triclosan injections and between the final triclosan injection and ^{3}H-E_2 administration, were chosen to exceed the 10.8±6.3 h terminal elimination half-life of dermally administered triclosan [6].

Blood and tissue samples were processed for liquid scintillation counting following previously published procedures used in this laboratory [24,37]. In brief, blood samples were centrifuged at 1,500 *g* for 10 min and 10 µl serum was added to a scintillation vial containing 5 ml Ultima Gold. Tissue samples were solubilized by adding 1 ml SOLVABLE to each vial and placing vials in a 50 °C water bath for 4–5 h until completely dissolved. Following the addition of 5 ml Ultima Gold, vials were agitated to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) for 5 min to eliminate background noise in the form of heat and luminescence. Radioactivity was measured for 5 min per vial and final adjusted estimates for the amount of radioactivity per sample in disintegrations per minute (dpm) were automatically

calculated by the accompanying Quanta-Smart software package. The dpm measures were then reported as either dpm/mg tissue or dpm/µl serum. A reported concentration of 1 dpm/mg tissue or 1 dpm/µl serum is equivalent to 1.52 pg/g tissue or 1.52 pg/ml serum, respectively.

2.4. Experiment 2: Measurement of ^{3}H-E_2 in cycling females after a single triclosan dose

At the onset of darkness, 20 cycling female mice were weighed (40.7 ± 4.1 g), individually housed, and each given a single sc injection of 0, 0.6, 1, or 2 mg triclosan (respectively 0.0 ± 0.0, 14.6 ± 1.7, 23.6 ± 2.9, or 53.9 ± 3.4 mg/kg) dissolved in 0.05 ml peanut oil (n=5 per dose). At 30 min after triclosan administration, each animal was given an ip injection of 1 µCi ^{3}H-E_2 (3.36 ng E_2) in 9 µl PBS. At 1 h after ^{3}H-E_2 administration, each animal was anesthetized with isoflurane. All other procedures, including perfusion, blood and tissue collection, sample processing, and scintillation counting were identical to those of Experiment 1. The 30-min latency between triclosan and ^{3}H-E_2 and the 1-h latency between ^{3}H-E_2 administration and tissue collection were chosen based on a previous study demonstrating an interaction between triclosan and ^{14}C-BPA [24]. A 1-h latency between ^{3}H-E_2 administration and tissue collection permits systemic distribution of ^{3}H-E_2 [38].

2.5. Experiment 3: Measurement of ^{3}H-E_2 in inseminated females after a single triclosan dose

We examined the influences of triclosan upon peri-implantation inseminated females given the potential relevance to blastocyst implantation failure. This should also produce less within-condition variance as endogenous E_2 dynamics are less variable than

among cycling females. Sexually naïve females were each paired with a male mouse aged 5–6 months and their hindquarters were inspected four times daily for the presence of a vaginal copulatory plug. Following detection of a copulatory plug, which marked gestation day 0, females were weighed (28.5 ± 1.6 g) and individually housed. At the onset of darkness on gestation day 3, females were each given a single sc injection of 0 or 2 mg triclosan (respectively 0.0 ± 0.0 or 69.9 ± 3.0 mg/kg) dissolved in 0.05 ml peanut oil. In Experiment 3A, each animal was given an ip injection of 1 µCi ^{3}H-E_2 (3.36 ng E_2) in 9 µl PBS at 1 h after triclosan administration (n=10 per dose). In Experiment 3B, each female was given ^{3}H-E_2 at 7 h after triclosan administration (n=10 per dose). In both Experiments 3A and 3B, each animal was anesthetized with isoflurane at 1 h after ^{3}H-E_2 administration. All other procedures, including perfusion, blood and tissue collection, sample processing, and scintillation counting were identical to those of Experiment 1. Here we also collected select brain areas including the olfactory bulbs, a caudal portion of the cerebellum, a section of the cortex, and a portion of the hypothalamic region (posterior to the optic chiasm and anterior to the pituitary stalk on the ventral surface of the brain). Neural tissue samples were processed and measured for radioactivity as were peripheral tissues in Experiment 1. We selected the 2 mg triclosan dose for use in peri-implantation females, as this dose was most effective in Experiments 1 and 2. The 1- and 7-h latencies between triclosan and ^{3}H-E_2 administrations were chosen to determine whether the effects of a single triclosan injection on ^{3}H-E_2 concentrations persisted for several hours after exposure. Neural tissue samples were collected for this experiment to

contrast female reproductive tissues with regions of the brain expressing high concentrations of ER.

2.6. Experiment 4: Measurement of urinary E_2 in cycling females given triclosan

Twenty female mice were weighed (29.9 ± 2.2 g) and individually placed in a Plexiglas apparatus measuring 21×15×13 (l×w×h) cm with a wire-mesh grid floor with squares measuring 0.5 cm^2. The wire-mesh grid floor was raised approximately 1 cm above a Teflon-coated stainless-steel surface covered with wax paper, which permitted non-invasive collection of urine. Mice were allowed to acclimate to the novel cages for 3 days prior to the start of the experiment. At the onset of darkness on the first day of the experiment, each female received a single sc injection of 0.05 ml peanut oil. Given the effects of a single triclosan exposure on ^{3}H-E_2 concentrations for up to 7 h in Experiment 3, urine was collected at 2, 4, 6, 8, 10, and 12 h post-injection. At the onset of darkness on day two, each mouse received a sc injection of 1 or 2 mg triclosan (respectively 33.5 ± 2.1 or 67.4 ± 5.8 mg/kg) dissolved in 0.05 ml peanut oil (n=10 per dose). Urine was once again collected at 2, 4, 6, 8, 10, and 12 h post-injection.

All urine samples were placed into labeled vials and frozen at –20 °C at the time of collection. Full procedures and validations for enzyme immunoassays for mouse urine were previously reported [39]. Cross-reactivities for anti-E_2 are: E_2 100%, estrone 3.3%, P_4 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids <0.1%. Urinary E_2 levels were considered with and without adjustment for urinary creatinine, which corrects for differential hydration and urinary concentration among animals, and reported as ng/mg creatinine and ng/ml urine, respectively.

2.7. Experiment 5: Measurement of urinary E_2 in inseminated females given triclosan

Thirty female mice were each paired with a male mouse and had their hindquarters inspected as in Experiment 3. Following detection of a copulatory plug, which marked gestation day 0, females were weighed (28.3 ± 1.5 g) and placed in a Plexiglas apparatus with a wire-mesh grid floor, then permitted to acclimate to the cages for 3 days. At the onset of darkness on gestation day 3, each female received a single sc injection of 0 or 2 mg triclosan (respectively 0.0 ± 0.0 or 71.0 ± 3.7 mg/kg) dissolved in 0.05 ml peanut oil (n=15 per dose). Urine was collected at 2, 4, 6, 8, 10, and 12 h post-injection. All other procedures, such as urine sample collection and storage, as well as enzyme immunoassay measurements, were identical to those of Experiment 4.

2.8. Statistical analyses

All statistical analyses were performed using the R software environment [40]. Data are presented as mean + standard error (SE). A comparison-wise error rate of $\alpha < 0.05$ was employed for all statistical tests. In Experiment 1, differences between ^{3}H-E_2 treatments were analyzed by univariate analysis of variance (ANOVA) for each tissue, with Holm-Bonferroni adjustments to correct for the number of tissues [41]. Significant effects in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons. Given that triclosan had especially strong influences in the uterus on the presence of ^{3}H-E_2 in Experiment 1 and the presence of ^{14}C-BPA in a previous study [24], we focused statistical comparisons on this tissue in subsequent experiments. In Experiments 2 and 3, concentrations of ^{3}H-E_2 in the uteri of animals given triclosan were compared to those of animals given an oil injection by two-tailed independent-samples t-test. In Experiments 4

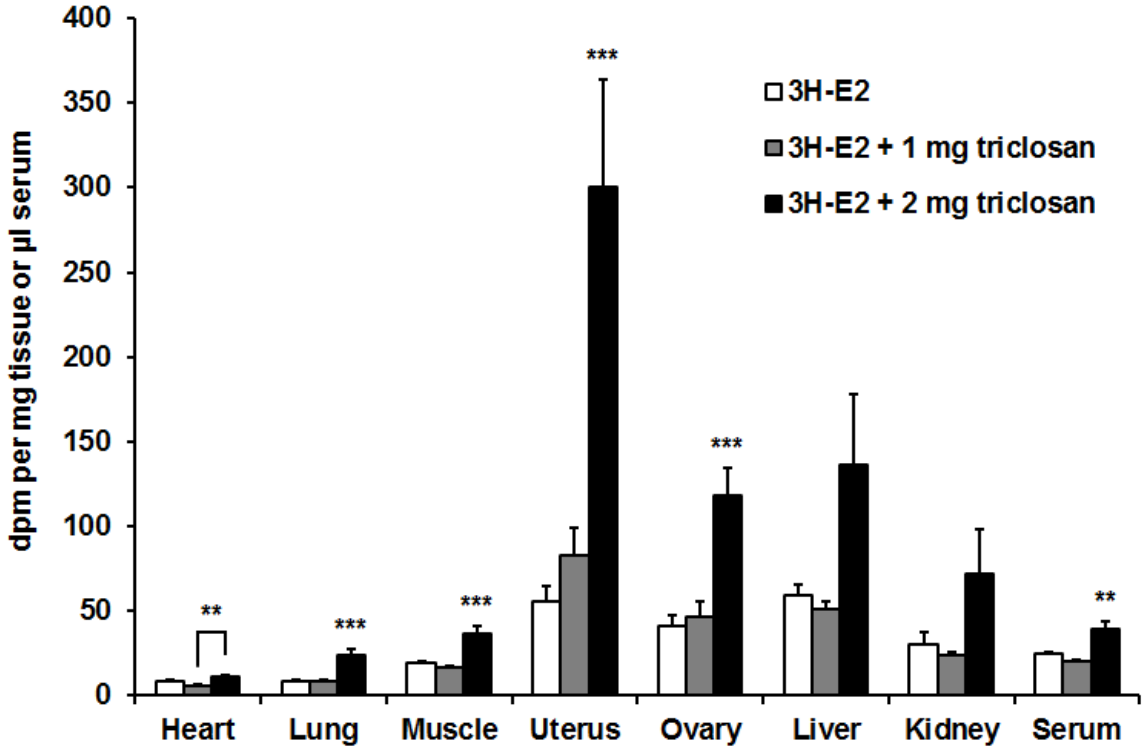
and 5, differences between urinary E_2 levels were analyzed by ANOVA comparing the effect of treatment on the volume-based and creatinine-corrected urinary E_2 concentrations. Significant effects in ANOVA were followed by t-tests at each urine collection time point, with Holm-Bonferroni adjustments to correct for the number of time points.

3. Results

3.1. Experiment 1: Measurement of ^{3}H-E_2 in cycling females after repeated triclosan doses

Radioactivity was measured in the peripheral tissues of cycling females that received daily sc injections of triclosan followed by an ip injection of 1 μCi ^{3}H-E_2 (Fig. 1). The impact of triclosan on ^{3}H-E_2 concentrations was most prominent in the reproductive tissues of female; mice given 2 mg triclosan had mean radioactivity levels that were 5.4 and 2.9 times higher than those of controls in the uterus and ovaries respectively. Statistical comparisons were made among the three treatments for each of eight tissues. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the heart, $F(2,18)=6.05$, $p=0.029$; lung, $F(2,18)=13.51$, $p=0.002$; muscle, $F(2,18)=17.23$, $p<0.001$; uterus, $F(2,18)=12.02$, $p=0.002$; ovary, $F(2,18)=14.82$, $p=0.001$; and serum, $F(2,18)=10.42$, $p=0.004$. Multiple comparisons revealed that the 2 mg triclosan dose differed from the 0 and 1 mg triclosan doses for the lung, muscle, uterus, ovary, and serum. The 2 mg triclosan dose also differed from the 1 mg triclosan dose for the heart.

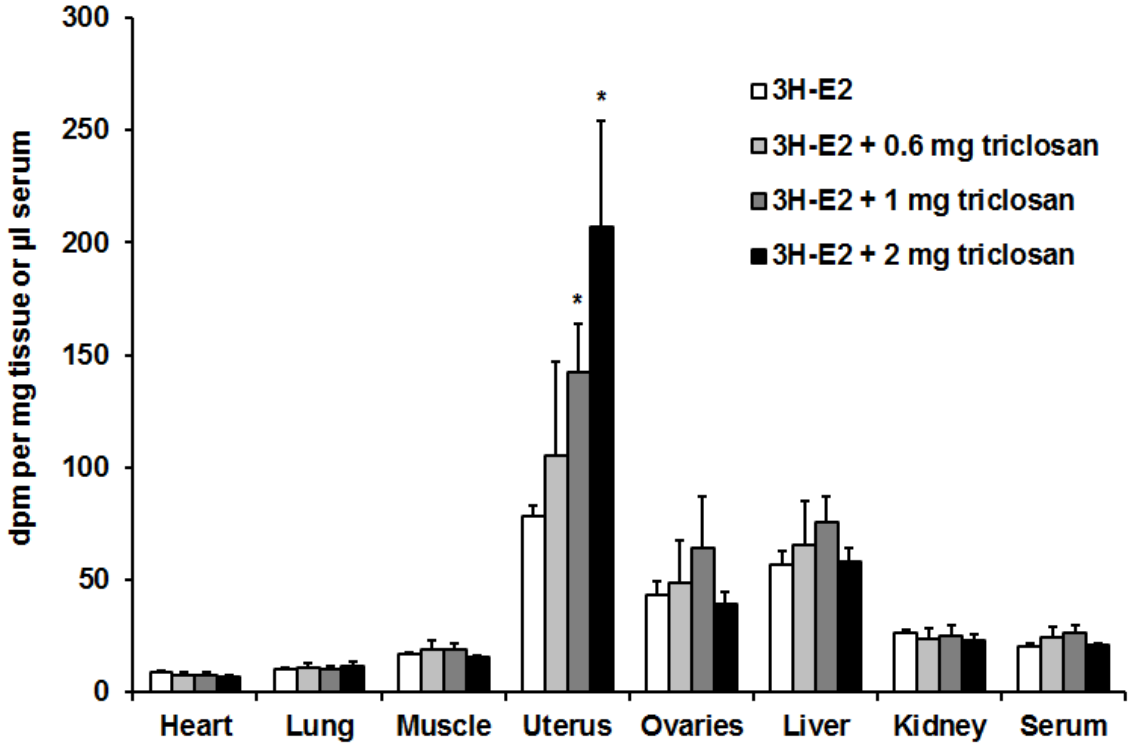
Fig. 1 Mean (+SE) concentration of radioactivity (dpm/mg tissue or dpm/µl serum) in the heart, lung, muscle, uterus, ovary, liver, kidney, and serum of cycling females following 7 daily sc injections of 0, 1, or 2 mg triclosan and subsequent ip injection of 1 µCi ^{3}H-E_2 (n=7 per dose) in Experiment 1. Unless otherwise noted, asterisks indicate that a condition differs significantly from the 0 mg and 1 mg triclosan treatments in the same tissue: ** $p < 0.01$; *** $p < 0.001$



3.2. Experiment 2: Measurement of ^{3}H-E_2 in cycling females after a single triclosan dose

Radioactivity was measured in the peripheral tissues of cycling females that received a single sc injection of triclosan followed by an ip injection of 1 µCi ^{3}H-E_2 (Fig. 2). Unlike repeated injections over several days, a single exposure of triclosan did not significantly modulate ^{3}H-E_2 concentrations in serum or peripheral tissues other than the uterus. Although ^{3}H-E_2 concentrations were greater in the uteri of mice given 2 mg triclosan, the magnitude of elevation was less than that in Experiment 1; mean radioactivity levels were 2.6 times (versus 5.4 times in Experiment 1) higher than those of controls in the uterus. Statistical comparisons were made between the 0 mg triclosan treatment and the 0.6, 1, or 2 mg triclosan treatments for the uterus. Two-tailed independent-samples t-test of treatment effects showed that, compared to animals given 0 mg triclosan, the level of radioactivity in the uterus was significantly greater in animals given 1 mg triclosan, $t(8)=2.90$, $p=0.020$, and in animals given 2 mg triclosan, $t(8)=2.70$, $p=0.027$.

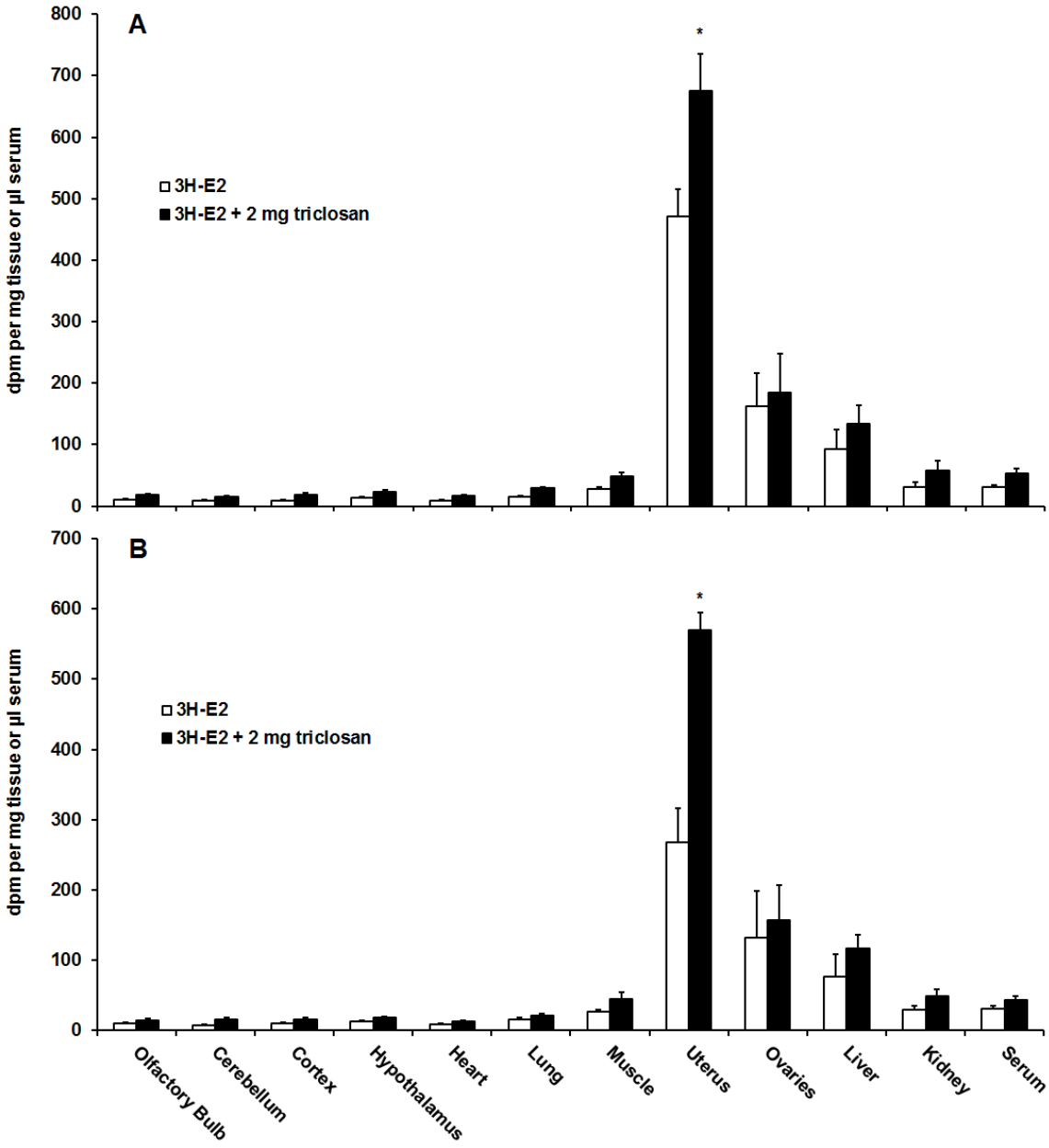
Fig. 2 Mean (+SE) concentration of radioactivity (dpm/mg tissue or dpm/µl serum) in the heart, lung, muscle, uterus, ovary, liver, kidney, and serum of cycling females following a single sc injection of 0, 0.6, 1, or 2 mg triclosan and subsequent ip injection of 1 µCi ^{3}H-E_2 (n=5 per dose) in Experiment 2. Significant difference from 0 mg treatment in the same tissue: * $p < 0.05$



3.3. Experiment 3: Measurement of ^{3}H-E_2 in inseminated females after a single triclosan dose

Radioactivity was measured in the peripheral tissues of inseminated females on gestation day 3 that received a single sc injection of triclosan followed by an ip injection of 1 μCi ^{3}H-E_2 (Fig. 3A and B). As in Experiments 1 and 2, the uterus showed the most pronounced effects; mean radioactivity levels were 1.4 and 2.1 times greater respectively at 1- and 7-h between triclosan and ^{3}H-E_2 exposures. Statistical comparisons were made between the 0 mg and 2 mg triclosan treatments for the uterus. Two-tailed independent-samples t-test of treatment effects showed that, when females were given ^{3}H-E_2 at 1 h after injection of vehicle or triclosan (Experiment 3A), the level of radioactivity in the uterus was significantly greater in animals given 2 mg triclosan, $t(18)=2.46$, $p=0.024$. When females were given ^{3}H-E_2 at 7 h after triclosan administration (Experiment 3B), the level of radioactivity in the uterus was once again significantly greater in animals given 2 mg triclosan, $t(18)=3.62$, $p=0.002$.

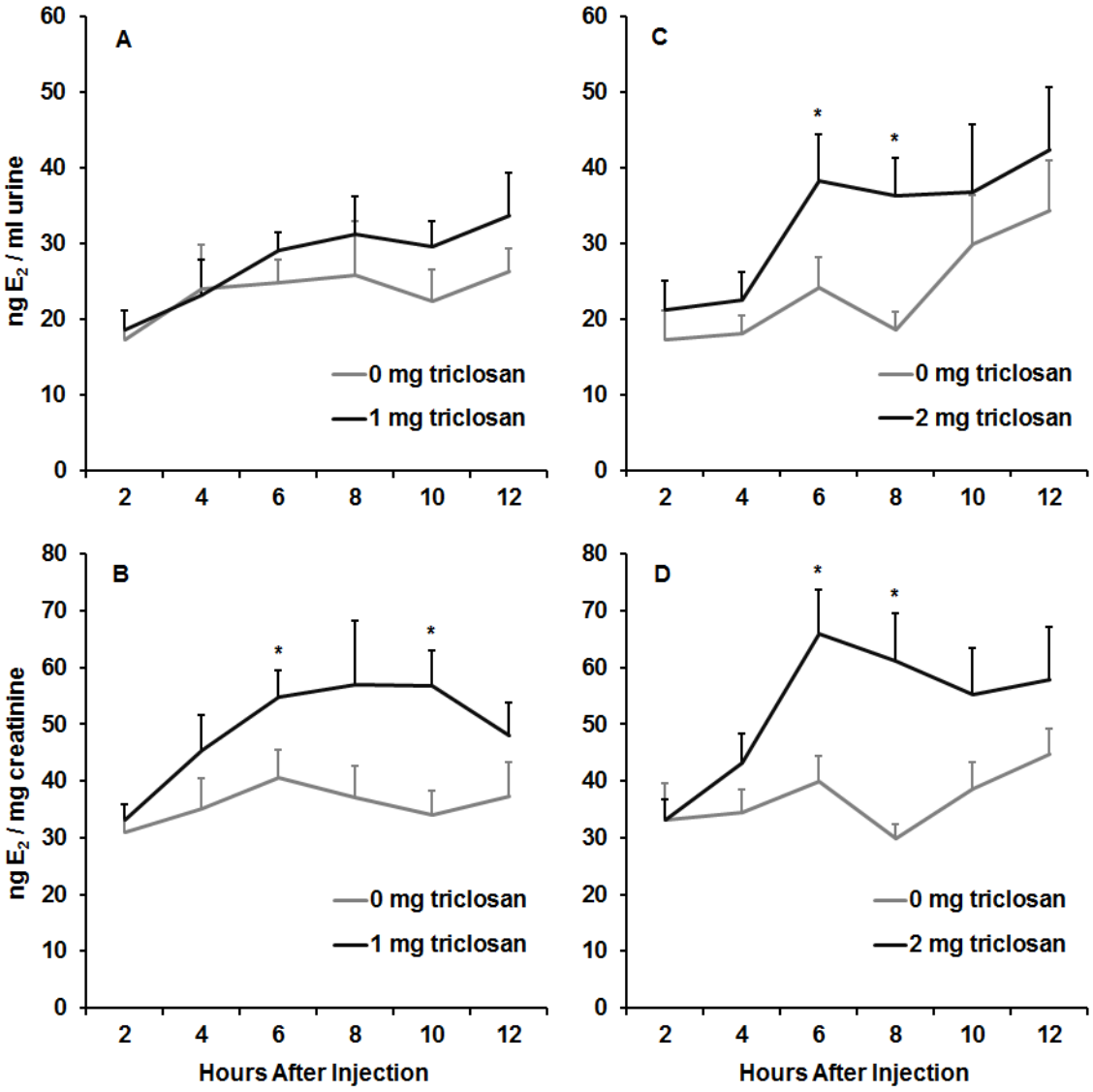
Fig. 3 Mean (+SE) concentration of radioactivity (dpm/mg tissue or dpm/µl serum) in the olfactory bulb, cerebellum, cortex, hypothalamus, heart, lung, muscle, uterus, ovary, liver, kidney, and serum of inseminated females on gestation day 3 following ip injection of 1 µCi ^{3}H-E_2 at (A) 1 h or (B) 7 h after a single sc injection of 0 or 2 mg triclosan (n=10 per dose per time point) in Experiment 3. Significant difference from 0 mg treatment in the same tissue: * $p < 0.05$


A
B
dpm per mg tissue or µl serum
3H-E2
3H-E2 + 2 mg triclosan
*
0
100
200
300
400
500
600
700
800
Olfactory Bulb
Cerebellum
Cortex
Hypothalamus
Heart
Lung
Muscle
Uterus
Ovaries
Liver
Kidney
Serum

3.4. Experiment 4: Measurement of urinary E_2 in cycling females given triclosan

Urinary E_2 levels of cycling females were measured the day prior and the day of a single injection of triclosan (Fig. 4A–D). Generally, the effect of triclosan on urinary E_2 concentrations was most prominent at around 6–10 h after injection. Repeated-measures ANOVA produced significant effects of treatment for the creatinine-corrected 1 mg triclosan dose, $F(1,53)=11.66$, $p=0.001$; the volume-based 2 mg triclosan dose, $F(1,53)=17.59$, $p<0.001$; and the creatinine-corrected 2 mg triclosan dose, $F(1,53)=26.09$, $p<0.001$. For the creatinine-corrected 1 mg triclosan dose, repeated-measures t-tests followed by Holm-Bonferroni correction produced significant effects of treatment at 6 h after injection, $t(9)=3.66$, $p=0.032$, and 10 h after injection, $t(6)=2.59$, $p=0.041$. For the volume-based 2 mg triclosan dose, significant effects of treatment were observed at 6 h after injection, $t(9)=3.38$, $p=0.040$, and 8 h after injection, $t(9)=3.91$, $p=0.021$. Similarly, for the creatinine-corrected 2 mg triclosan dose, significant effects of treatment were observed at 6 h after injection, $t(9)=4.12$, $p=0.013$, and 8 h after injection, $t(9)=4.23$, $p=0.013$.

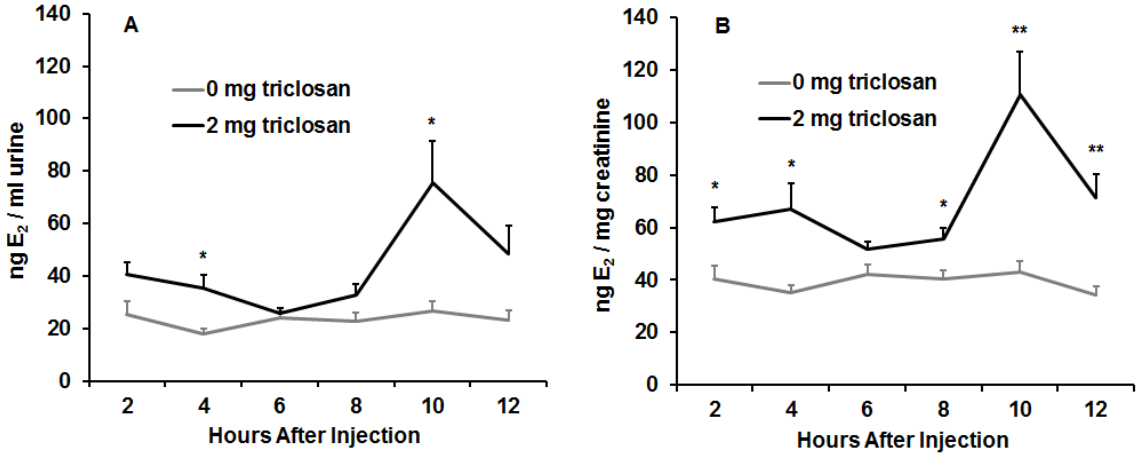
Fig. 4 Mean (+SE) level of urinary E_2 (ng E_2 / ml urine and ng E_2 / mg creatinine) before and after a single sc injection of (A, B) 1 mg or (C, D) 2 mg triclosan in cycling females (n=10 per dose) in Experiment 4. Significant difference from 0 mg treatment at the same time point: * $p < 0.05$



3.5. Experiment 5: Measurement of urinary E_2 in inseminated females given triclosan

Urinary E_2 levels of inseminated females on gestation day 3 were measured after a single sc injection of 0 or 2 mg triclosan (Fig. 5A and B). The effect of triclosan on urinary E_2 concentrations was evident throughout the 2–12 h collection window; however, the effect was most prominent at around 10–12 h after injection. ANOVA produced significant effects of treatment for the volume-based measures, $F(1,164)=27.87$, $p<0.001$, and the creatinine-corrected measures, $F(1,164)=59.25$, $p<0.001$. For the volume-based 2 mg triclosan dose, t-tests followed by Holm-Bonferroni correction produced significant effects of treatment at 4 h after injection, $t(27)=3.39$, $p=0.013$, and 10 h after injection, $t(28)=2.92$, $p=0.034$. For the creatinine-corrected 2 mg triclosan dose, significant effects of treatment were observed at 2 h after injection, $t(28)=3.00$, $p=0.017$; 4 h after injection, $t(27)=3.35$, $p=0.010$; 8 h after injection, $t(27)=2.90$, $p=0.017$; 10 h after injection, $t(28)=3.98$, $p=0.002$; and 12 h after injection, $t(26)=4.09$, $p=0.002$.

Fig. 5 Mean (+SE) level of urinary E_2 (A: ng E_2 / ml urine, B: ng E_2 / mg creatinine) following a single sc injection of 0 or 2 mg triclosan in inseminated females on gestation day 3 (n=15 per dose) in Experiment 5. Significant difference from 0 mg treatment at the same time point: * $p < 0.05$; ** $p < 0.01$



4. Discussion

To the best of our knowledge, these data are the first to show that triclosan modulates the concentrations of exogenous and endogenous E_2 *in vivo*. We have shown this using two strategies. First, we found enhanced uptake of ^{3}H-E_2 in the uterus and other tissues of female mice that received triclosan. Second, after triclosan administration, we observed an elevation in endogenous E_2 as reflected in urinary measures. We measured urinary E_2 given evidence that enzyme immunoassay cannot reliably quantify E_2 in mouse serum due to extensive binding to carrying molecules in blood [42]. Moreover, whereas previous evidence found very low concentrations of estrogen conjugates in mouse urine [39], unconjugated E_2 is abundant there and it generally reflects systemic trends [39,43–46].

There are several potential mechanisms through which triclosan and E_2 could interact, including actions at estrogen receptors, blood binding factors, and enzymes involved in steroid metabolism. Although there is some evidence that triclosan can interact with estrogen receptors, such action is competitive with binding of E_2 [19,20]. That would suggest an opposite effect to the finding in our data that triclosan magnified ^{3}H-E_2 presence in the uterus and other tissues. Similarly, competition for blood binding factors would presumably reduce E_2 presence in serum and tissues. A strong possibility is that our findings arise from triclosan's actions on sulfotransferase. In *in vitro* human liver fractions, low nM to µM concentrations of triclosan can potently inhibit estrone and E_2 sulfonation by non-competitively interacting with various sulfotransferase isoforms, including SULT 1E1, 1B1, and 1A1 [21,22]. Given that a considerable portion of

triclosan present in the body is localized within the liver [14], we suggest that triclosan may interact with hepatic sulfotransferases to inhibit the conjugation of exogenous and endogenous E_2.

We found that triclosan magnified radioactivity in the heart, lungs, muscle, uterus, ovaries, and blood serum of females given ^{3}H-E_2. The chosen dose of ^{3}H-E_2 represented just a small fraction of the animals' endogenous E_2 [35]. The interaction of triclosan with exogenous ^{3}H-E_2 may be relevant to human estrogen supplementation, albeit with some caveats. Whereas we injected ^{3}H-E_2 ip, human estrogen supplementation usually involves E_2 derivatives such as ethinyl estradiol [47] administered via an oral pill, transdermal patch, or vaginal ring [47]. However, triclosan can potentiate the estrogenic effects of ethinyl estradiol in rats [17,18]. In all experiments involving ^{3}H-E_2 administration, the uterus showed the highest concentrations in vehicle-treated animals. In addition, we observed the greatest impact of triclosan exposure on the uterus, with the 2-mg triclosan dose elevating concentrations by 140 to 540% across experiments. Whereas repeated triclosan doses increased concentrations of ^{3}H-E_2 in serum and most peripheral tissues, only concentrations in the uterus were significantly elevated following a single triclosan injection. The localization of ^{3}H-E_2 to the female reproductive tract is consistent with the high expression of ERα and ERβ in these tissues [48,49]. In humans, higher household incomes correlate with higher urinary triclosan concentrations [8] and greater use of hormonal contraceptives [50]; study of the effects of triclosan on estrogen supplementation in contraceptives and hormone-replacement therapy may be warranted.

We also showed elevated levels of endogenous urinary E_2 following triclosan administration. This effect was greater in recently inseminated females, spanning between 2 and 12 h after triclosan injection in the creatinine-corrected measures, compared to between 6 and 10 h in cycling females. This may be due to the reduced variance in urinary E_2 measures among inseminated females. Even slight elevations in E_2 levels can lead to adverse health and reproductive outcomes in human and other mammalian females. In humans, it is well established that persistently elevated E_2 levels correlate with an increased risk of breast [31,51], endometrial [32], and ovarian cancer [33]. In mice, elevated E_2 levels can disrupt pregnancy by preventing intrauterine blastocyst implantation [45]. These data are also consistent with the finding that triclosan can have estrogenic effects in the uterus, increasing uterine mass and expression of genes upregulated by natural estrogens [52].

At doses as low as 1 or 2 mg triclosan, we showed elevated concentrations of exogenous ^{3}H-E_2 in reproductive tissues and natural E_2 in urine. The doses of triclosan in the present study are arguably higher than those to which most humans are exposed. Urinary measures from large samples of the U.S. population indicate widespread exposure to triclosan [8,9], but there are few published attempts to quantify this exposure. One study estimated that the average daily intake of triclosan in U.S. individuals is 350 µg [53], which is equivalent to 5 µg/kg for a 70 kg adult. No estimate of occupational exposure levels exists, which may be important considering the wide variance in human urinary triclosan concentrations, where the highest measured concentration was nearly 300 times greater than the geometric mean [8].

Our data are concordant with a mechanism whereby the non-competitive binding of triclosan to sulfotransferase and/or other metabolic enzymes inhibits E_2 metabolism. Our data do not support interaction of triclosan with ER as an agonist or antagonist, or interaction of triclosan with blood binding factors, which would produce opposing effects to those observed here. A number of environmental chemicals and their metabolites with chemical structures similar to that of triclosan have been shown to inhibit estrogen sulfotransferase, including polychlorinated biphenyls [54,55], polyhalogenated hydrocarbons [56], brominated flame retardants [57], and parabens [58]. Accordingly, concurrent exposure to multiple toxicants might lead to summation or other interactions of their effects. Given the potential carcinogenic and adverse reproductive influences of excessive estrogenic activity, further research is needed on such interactions and indirect estrogenic influences.

Acknowledgements

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References

[1] R.J. Heath, J.R. Rubin, D.R. Holland, E. Zhang, M.E. Snow, C.O. Rock, Mechanism of triclosan inhibition of bacterial fatty acid synthesis, J. Biol. Chem. 274 (1999) 11110–11114. doi:10.1074/jbc.274.16.11110.

[2] J.-L. Fang, R.L. Stingley, F.A. Beland, W. Harrouk, D.L. Lumpkins, P. Howard, Occurrence, efficacy, metabolism, and toxicity of triclosan, J. Environ. Sci. Health Part C 28 (2010) 147–171. doi:10.1080/10590501.2010.504978.

[3] J.V. Rodricks, J.A. Swenberg, J.F. Borzelleca, R.R. Maronpot, A.M. Shipp, Triclosan: A critical review of the experimental data and development of margins of safety for consumer products, Crit. Rev. Toxicol. 40 (2010) 422–484. doi:10.3109/10408441003667514.

[4] E.N. Perencevich, M.T. Wong, A.D. Harris, National and regional assessment of the antibacterial soap market: a step toward determining the impact of prevalent antibacterial soaps, Am. J. Infect. Control 29 (2001) 281–283.

[5] T. Moss, D. Howes, F.M. Williams, Percutaneous penetration and dermal metabolism of triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether), Food Chem. Toxicol. 38 (2000) 361–370. doi:10.1016/S0278-6915(99)00164-7.

[6] C. Queckenberg, J. Meins, B. Wachall, O. Doroshyenko, D. Tomalik-Scharte, B. Bastian, M. Abdel-Tawab, U. Fuhr, Absorption, pharmacokinetics, and safety of triclosan after dermal administration, Antimicrob. Agents Chemother. 54 (2010) 570–572. doi:10.1128/AAC.00615-09.

[7] G. Sandborgh-Englund, M. Adolfsson-Erici, G. Odham, J. Ekstrand, Pharmacokinetics of triclosan following oral ingestion in humans, J. Toxicol. Environ. Health A. 69 (2006) 1861–1873. doi:10.1080/15287390600631706.

[8] A.M. Calafat, X. Ye, L.-Y. Wong, J.A. Reidy, L.L. Needham, Urinary concentrations of triclosan in the U.S. population: 2003–2004, Environ. Health Perspect. 116 (2008) 303–307. doi:10.1289/ehp.10768.

[9] C. Han, Y.-H. Lim, Y.-C. Hong, Ten-year trends in urinary concentrations of triclosan and benzophenone-3 in the general U.S. population from 2003 to 2012, Environ. Pollut. 208 (2016) 803–810. doi:10.1016/j.envpol.2015.11.002.

[10] M. Allmyr, F. Harden, L.-M.L. Toms, J.F. Mueller, M.S. McLachlan, M. Adolfsson-Erici, G. Sandborgh-Englund, The influence of age and gender on triclosan concentrations in Australian human blood serum, Sci. Total Environ. 393 (2008) 162–167. doi:10.1016/j.scitotenv.2007.12.006.

[11] T. Geens, H. Neels, A. Covaci, Sensitive and selective method for the determination of bisphenol-A and triclosan in serum and urine as pentafluorobenzoate-derivatives using GC–ECNI/MS, J. Chromatogr. B. 877 (2009) 4042–4046. doi:10.1016/j.jchromb.2009.10.017.

[12] M. Allmyr, M. Adolfsson-Erici, M.S. McLachlan, G. Sandborgh-Englund, Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products, Sci. Total Environ. 372 (2006) 87–93. doi:10.1016/j.scitotenv.2006.08.007.

[13] L.-M.L. Toms, M. Allmyr, J.F. Mueller, M. Adolfsson-Erici, M. McLachlan, J. Murby, F.A. Harden, Triclosan in individual human milk samples from Australia, Chemosphere 85 (2011) 1682–1686. doi:10.1016/j.chemosphere.2011.08.009.

[14] T. Geens, H. Neels, A. Covaci, Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain, Chemosphere 87 (2012) 796–802. doi:10.1016/j.chemosphere.2012.01.002.

[15] J.-Y. Kim, B.-R. Yi, R.-E. Go, K.-A. Hwang, K.-H. Nam, K.-C. Choi, Methoxychlor and triclosan stimulates ovarian cancer growth by regulating cell cycle- and apoptosis-related genes via an estrogen receptor-dependent pathway, Environ. Toxicol. Pharmacol. 37 (2014) 1264–1274. doi:10.1016/j.etap.2014.04.013.

[16] H.-R. Lee, K.-A. Hwang, K.-H. Nam, H.-C. Kim, K.-C. Choi, Progression of breast cancer cells was enhanced by endocrine-disrupting chemicals, triclosan and octylphenol, via an estrogen receptor-dependent signaling pathway in cellular and mouse xenograft models, Chem. Res. Toxicol. 27 (2014) 834–842. doi:10.1021/tx5000156.

[17] T.E. Stoker, E.K. Gibson, L.M. Zorrilla, Triclosan exposure modulates estrogen-dependent responses in the female Wistar rat, Toxicol. Sci. 117 (2010) 45–53. doi:10.1093/toxsci/kfq180.

[18] G.W. Louis, D.R. Hallinger, T.E. Stoker, The effect of triclosan on the uterotrophic response to extended doses of ethinyl estradiol in the weanling rat, Reprod. Toxicol. 36 (2013) 71–77. doi:10.1016/j.reprotox.2012.12.001.

[19] R.H. Gee, A. Charles, N. Taylor, P.D. Darbre, Oestrogenic and androgenic activity of triclosan in breast cancer cells, J. Appl. Toxicol. 28 (2008) 78–91. doi:10.1002/jat.1316.

[20] N.D. Henry, P.A. Fair, Comparison of *in vitro* cytotoxicity, estrogenicity and anti-estrogenicity of triclosan, perfluorooctane sulfonate and perfluorooctanoic acid: *In vitro* cytotoxicity and estrogenicity of emerging contaminants, J. Appl. Toxicol. 33 (2013) 265–272. doi:10.1002/jat.1736.

[21] M.O. James, W. Li, D.P. Summerlot, L. Rowland-Faux, C.E. Wood, Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta, Environ. Int. 36 (2010) 942–949. doi:10.1016/j.envint.2009.02.004.

[22] M. James, S. Ambadapadi, C. Falany, Triclosan inhibits the activity of expressed human sulfotransferases (SULTs) towards their diagnostic substrates, FASEB J. 29 (2015) 622 (Abstract).

[23] L. Wang, C.N. Falany, M.O. James, Triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions, Drug Metab. Dispos. 32 (2004) 1162–1169. doi:10.1124/dmd.104.000273.

[24] T. Pollock, B. Tang, D. deCatanzaro, Triclosan exacerbates the presence of ^{14}C-bisphenol A in tissues of female and male mice, Toxicol. Appl. Pharmacol. 278 (2014) 116–123. doi:10.1016/j.taap.2014.04.017.

[25] R.G. Berger, W.G. Foster, D. deCatanzaro, Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of

estrogen and progesterone receptor expression in mice, Reprod. Toxicol. 30 (2010) 393–400. doi:10.1016/j.reprotox.2010.06.006.

[26] R.G. Berger, J. Shaw, D. deCatanzaro, Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol, Reprod. Toxicol. 26 (2008) 94–99. doi:10.1016/j.reprotox.2008.06.007.

[27] R.G. Berger, T. Hancock, D. deCatanzaro, Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice, Reprod. Toxicol. 23 (2007) 138–144. doi:10.1016/j.reprotox.2006.09.005.

[28] E.D. Borman, W.G. Foster, M.K.E. Greenacre, C.C. Muir, D. deCatanzaro, Stress lowers the threshold dose at which bisphenol A disrupts blastocyst implantation, in conjunction with decreased uterine closure and e-cadherin, Chem. Biol. Interact. 237 (2015) 87–95. doi:10.1016/j.cbi.2015.05.012.

[29] B.R. Crawford, D. deCatanzaro, Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A, Reprod. Toxicol. 34 (2012) 607–613. doi:10.1016/j.reprotox.2012.09.008.

[30] D. deCatanzaro, Sex steroids as pheromones in mammals: The exceptional role of estradiol, Horm. Behav. 68 (2015) 103–116. doi:10.1016/j.yhbeh.2014.08.003.

[31] V. Beral, Breast cancer and hormone-replacement therapy in the Million Women Study, The Lancet 362 (2003) 419–427. doi:10.1016/S0140-6736(03)14065-2.

[32] V. Beral, Endometrial cancer and hormone-replacement therapy in the Million Women Study, The Lancet 365 (2005) 1543–1551. doi:10.1016/S0140-6736(05)66455-0.

[33] V. Beral, Ovarian cancer and hormone replacement therapy in the Million Women Study, The Lancet 369 (2007) 1703–1710. doi:10.1016/S0140-6736(07)60534-0.

[34] W. Ma, H. Song, S.K. Das, B.C. Paria, S.K. Dey, Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation, Proc. Natl. Acad. Sci. 100 (2003) 2963–2968.

[35] A.C. Guzzo, T. Pollock, D. deCatanzaro, Transfer of [^{3}H]estradiol-17β and [^{3}H]progesterone from conspecifics to cohabiting female mice, J. Endocrinol. 217 (2013) 1–10. doi:10.1530/JOE-12-0279.

[36] J.B. Thorpe, D. deCatanzaro, Oestradiol treatment restores the capacity of castrated males to induce both the Vandenbergh and the Bruce effects in mice (*Mus musculus*), Reproduction 143 (2012) 123–132. doi:10.1530/REP-11-0251.

[37] T. Pollock, D. deCatanzaro, Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses, Reprod. Toxicol. 49 (2014) 145–154. doi:10.1016/j.reprotox.2014.08.005.

[38] A.C. Guzzo, J. Jheon, F. Imtiaz, D. deCatanzaro, Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (Mus musculus), Reproduction 143 (2012) 539–548. doi:10.1530/REP-11-0375.

[39] C. Muir, E. Spironello-Vella, N. Pisani, D. deCatanzaro, Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples

from male and female mice, Horm. Metab. Res. 33 (2001) 653–658. doi:10.1055/s-2001-18692.

[40] R Core Team, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2015. http://www.R-project.org/.

[41] S. Holm, A simple sequentially rejective multiple test procedure, Scand. J. Stat. 6 (1979) 65–70.

[42] D.J. Haisenleder, A.H. Schoenfelder, E.S. Marcinko, L.M. Geddis, J.C. Marshall, Estimation of estradiol in mouse serum samples: Evaluation of commercial estradiol immunoassays, Endocrinology 152 (2011) 4443–4447. doi:10.1210/en.2011-1501.

[43] D. deCatanzaro, C. Muir, E. Beaton, M. Jetha, K. Nadella, Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination, Reproduction 126 (2003) 407–414.

[44] D. deCatanzaro, C. Muir, E.A. Beaton, M. Jetha, Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice, Steroids 69 (2004) 687–696. doi:10.1016/j.steroids.2004.07.002.

[45] J.B. Thorpe, P.S. Burgess, M. Sadkowski, D. deCatanzaro, Estrogen–progesterone balance in the context of blastocyst implantation failure induced by predator stress, Psychoneuroendocrinology 38 (2013) 3048–3056. doi:10.1016/j.psyneuen.2013.09.001.

[46] J. Thorpe, K. Gould, E. Borman, D. deCatanzaro, Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*), Horm. Metab. Res. 46 (2014) 211–218. doi:10.1055/s-0033-1363958.

[47] M.W. van den Heuvel, A.J.M. van Bragt, A.K.M. Alnabawy, M.C.J. Kaptein, Comparison of ethinylestradiol pharmacokinetics in three hormonal contraceptive formulations: the vaginal ring, the transdermal patch and an oral contraceptive, Contraception 72 (2005) 168–174. doi:10.1016/j.contraception.2005.03.005.

[48] J.F. Couse, J. Lindzey, K. Grandien, J. Gustafsson, K.S. Korach, Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse, Endocrinology 138 (1997) 4613–4621.

[49] G.G. Kuiper, B.O. Carlsson, K.A.J. Grandien, E. Enmark, J. Häggblad, S. Nilsson, J.-A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β, Endocrinology 138 (1997) 863–870.

[50] W.D. Mosher, J. Jones, Use of contraception in the United States: 1982-2008, U.S. Dept. of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics, Hyattsville, Md, 2010.

[51] J.D. Yager, N.E. Davidson, Estrogen carcinogenesis in breast cancer, N. Engl. J. Med. 354 (2006) 270–282.

[52] E.-M. Jung, B.-S. An, K.-C. Choi, E.-B. Jeung, Potential estrogenic activity of triclosan in the uterus of immature rats and rat pituitary GH3 cells, Toxicol. Lett. 208 (2012) 142–148. doi:10.1016/j.toxlet.2011.10.017.

[53] W. Nazaroff, C.J. Weschler, J.C. Little, E.A.C. Hubal, Intake to production ratio: a measure of exposure intimacy for manufactured chemicals, Environ. Health Perspect. 120 (2012) 1678–1683.

[54] M.H. Kester, S. Bulduk, D. Tibboel, W. Meinl, H. Glatt, C.N. Falany, M.W. Coughtrie, A. Bergman, S.H. Safe, G.G. Kuiper, A.G. Schuur, A. Brouwer, T.J. Visser, Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: A novel pathway explaining the estrogenic activity of PCBs, Endocrinology 141 (2000) 1897–1900.

[55] L. Wang, M. James, Sulfonation of 17β-estradiol and inhibition of sulfotransferase activity by polychlorobiphenylols and celecoxib in channel catfish, Ictalurus punctatus, Aquat. Toxicol. 81 (2007) 286–292. doi:10.1016/j.aquatox.2006.12.011.

[56] M.H. Kester, S. Bulduk, H. van Toor, D. Tibboel, W. Meinl, H. Glatt, C.N. Falany, M.W. Coughtrie, A.G. Schuur, A. Brouwer, others, Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters, J. Clin. Endocrinol. Metab. 87 (2002) 1142–1150.

[57] R.A. Gosavi, G.A. Knudsen, L.S. Birnbaum, L.C. Pedersen, Mimicking of estradiol binding by flame retardants and their metabolites: A crystallographic analysis, Environ. Health Perspect. 121 (2013) 1194–1199. doi:10.1289/ehp.1306902.

[58] J.J. Prusakiewicz, H.M. Harville, Y. Zhang, C. Ackermann, R.L. Voorman, Parabens inhibit human skin estrogen sulfotransferase activity: Possible link to paraben estrogenic effects, Toxicology 232 (2007) 248–256. doi:10.1016/j.tox.2007.01.010.

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Chapter 5

Butyl paraben and propyl paraben modulate bisphenol A and estradiol concentrations in female and male mice

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Authors' Contributions

Tyler Pollock: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

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Abstract

People are routinely exposed to the antimicrobial preservatives butyl paraben (BP) and propyl paraben (PP), as well as the monomer of polycarbonate plastics, bisphenol A (BPA). These chemicals are reliably detected in human urine and potentially interact. We investigated whether BP or PP exposure can modulate the concentrations of ^{14}C-BPA and 17β-estradiol (E_2). Female and male CF1 mice were each given a subcutaneous injection of oil containing 0 (vehicle), 1, 3, or 9 mg BP or PP, then given a dietary supplement containing 50 µg/kg ^{14}C-BPA. Radioactivity was measured in tissues through liquid scintillation counting. Significantly elevated ^{14}C-BPA concentrations were observed following BP treatment in blood serum of both sexes, as well as the lungs, uterus, and ovaries of females and the testes and epididymides of males. Treatment with PP significantly elevated ^{14}C-BPA concentrations in the uterus only. In another experiment, female and male CF1 mice were each injected with vehicle, 3 mg BP, or 3 mg PP, and E_2 was measured in urine 2–12 h later. Whereas PP did not affect E_2, BP significantly elevated E_2 6–10 h after injection in females and 8 h after injection in males. These data indicate that BP and PP can alter the pharmacokinetics of BPA *in vivo*, and that BP can modulate E_2 concentrations. These results are consistent with evidence that parabens inhibit enzymes that are critical for BPA and E_2 metabolism, and demonstrate the importance of considering concurrent exposure to multiple chemicals when determining regulatory exposure limits.

1. Introduction

Parabens (*p*-hydroxybenzoic acid esters) are used as antimicrobial preservatives in cosmetics, personal care products, pharmaceuticals, foods, and beverages (Andersen, 2008). Their ubiquitous use is attributed to worldwide regulatory acceptance, broad inertness, and low cost (Soni et al., 2005). Dermal exposure from cosmetics and personal care products is the primary route of human exposure (Błędzka et al., 2014), whereas dietary consumption from foods, beverages, and pharmaceuticals is the secondary route (Błędzka et al., 2014). Based on the 2005–2006 U.S. National Health and Nutrition Survey (NHANES), methyl paraben (MP) and propyl paraben (PP) were detected in 99.1% and 92.7% of the population at concentrations of 1.0–17,300 µg/L urine and 0.2–7,210 µg/L urine respectively (Calafat et al., 2010). Butyl paraben (BP) and ethyl paraben (EP) were less common, being detected in 47% and 42.4% of the population at concentrations of 0.2–1,240 µg/L urine and 1.0–1,110 µg/L urine respectively (Calafat et al., 2010). Similar patterns were observed in NHANES data from 2007–2012 (CDC, 2015) and in other biomonitoring studies (Kang et al., 2016; Smith et al., 2012). Patterns in urinary paraben concentrations were mostly concordant with human exposure estimates; MP and PP were highest at 0.79–1.61 mg/kg/day and 0.34–0.80 mg/kg/day respectively, whereas BP was lower at 0.002–0.02 mg/kg/day (Cowan-Ellsberry and Robison, 2009).

Parabens have been identified as chemicals of concern after *in vitro* studies demonstrated that they can bind estrogen receptor (ER) α and β (Blair et al., 2000; Byford et al., 2002; Gomez et al., 2005; Miller et al., 2001; Watanabe et al., 2013). Affinity for

ER is several orders of magnitude lower than that of 17β-estradiol (E_2); BP is the most potent with approximately 10,000-fold lower potency than E_2 (Routledge et al., 1998). Concern has been raised after parabens were detected in human breast tumors (Darbre et al., 2004) and evidence that they could increase proliferation of MCF-7 human breast cancer cells *in vitro* (Charles and Darbre, 2013). *In vivo* studies have shown that parabens can induce positive responses in rodent uterotrophic assays (Lemini et al., 2004; Routledge et al., 1998), induce estrogenic histological changes in the uterus (Lemini et al., 2004), and disrupt male reproductive parameters (Oishi, 2002a, 2002b; Zhang et al., 2016). Parabens can interact with other environmental chemicals; one study showed that BP and diethylhexyl phthalate could disrupt ovarian steroidogenesis, leading to attenuated E_2 output, only when administered concurrently (Guerra et al., 2016). Parabens can also inhibit enzymes involved in the metabolism of estrogens, including sulfotransferase (SULT), cytochrome P450 (CYP), and UDP-glucuronosyltransferase (UGT) (Dumas and Diorio, 2011). Parabens act as competitive inhibitors of SULT1A1 (Prusakiewicz et al., 2007) as well as numerous CYP (Ozaki et al., 2016) and UGT (Abbas et al., 2010) isoforms. These actions could disrupt estrogen homeostasis and thereby produce indirect estrogenic effects.

We previously demonstrated *in vivo* interaction of triclosan, an antimicrobial agent found in personal care products, with bisphenol A (BPA) and E_2. Female mice given 1–2 mg triclosan showed greater concentrations of exogenous ^{3}H-E_2 in the uterus and natural E_2 in urine compared to vehicle-treated animals (Pollock et al., 2016). Similarly, female and male mice given 0.6–18 mg triclosan showed greater

concentrations of ^{14}C-BPA in blood serum and reproductive and other tissues (Pollock et al., 2014). Blastocyst implantation in the uterus of inseminated females could also be disrupted by approximately 200 mg BPA/kg/day, 523 mg triclosan/kg/day, or a lower dose combination of 122 mg BPA/kg/day + 262 mg triclosan/kg/day for 3 days (Berger et al., 2010, 2008, 2007; Crawford and deCatanzaro, 2012). These findings are consistent with *in vitro* evidence that triclosan can non-competitively inhibit SULT and competitively inhibit UGT activities towards BPA and E_2 (James et al., 2015, 2010; Wang et al., 2004).

Humans are frequently exposed to multiple potential endocrine-disrupting chemicals. Here we sought to determine the capacity of certain parabens to modulate the pharmacokinetics of BPA and E_2 *in vivo*. We selected BP and PP for these studies, as the Scientific Committee on Consumer Products of the European Commission stated that, although MP and EP are considered safe, there is not yet sufficient data to complete a safety assessment for BP and PP (SCCP, 2008; SCCS, 2013). In comparison to BP and PP, BPA is a more established environmental estrogen (Rochester, 2013; Seachrist et al., 2016; Ziv-Gal and Flaws, 2016), whereas E_2 is the most potent natural estrogen (Blair et al., 2000; Kuiper et al., 1997). In rodents, BPA primarily undergoes phase II conjugative metabolism via interaction with UGT and SULT (Inoue et al., 2016; Kurebayashi et al., 2010; Yalcin et al., 2016; Zalko et al., 2003). In addition to UGT and SULT, estrogen concentrations are also regulated by phase I oxidative metabolism via CYP (Dumas and Diorio, 2011). Given inhibitory actions of parabens on these enzymes (Abbas et al., 2010; Ozaki et al., 2016; Prusakiewicz et al., 2007), we hypothesized that they would elevate

BPA and E_2 concentrations in female and male mice. Here we measured the impact of BP or PP injection on concentrations of dietary ^{14}C-BPA in blood serum and tissues of mice. We also measured the impact of BP or PP injection on concentrations of urinary E_2, as E_2 in mouse urine reliably reflects systemic trends in estrogen activity (deCatanzaro et al., 2004, 2003, Thorpe et al., 2014, 2013) and can be collected non-invasively at multiple time-points.

2. Materials and methods

2.1. Animals and housing

Female (28.4 ± 2.2 g) and male (38.9 ± 5.1 g) CF1 mice aged 2.5–3.5 months were obtained from Charles River (Kingston, NY). Diestrous females were selected for use in experiments from a colony of mice with regular estrous cycles by vaginal cytology using published procedures (Byers et al., 2012). Animals were housed in polypropylene cages measuring 28×16×11 (l×w×h) cm with *ad libitum* access to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University.

2.2. Chemicals and materials

BP (CAS 94-26-8, butyl 4-hydroxybenzoate, ≥99% purity), PP (CAS 94-13-3, propyl 4-hydroxybenzoate, ≥99% purity), 17β-E_2 (≥98% purity), and creatinine standards were obtained from Sigma-Aldrich, St. Louis, MO. ^{14}C-BPA ([ring-[^{14}C](U)]-BPA, in

ethanol, 0.1 mCi/ml, 60 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, 8 ml midi-vial scintillation vials were obtained from PerkinElmer, Waltham, MA. 17β-E_2 antibodies and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA.

2.3. Experimental design and dosing

This research followed previously published procedures used in this laboratory (Pollock et al., 2016, 2014). Mice were weighed, individually housed, and each given a dietary supplement of 1 g peanut butter. About 14–16 h later, beginning at the onset of darkness (9:00 AM) on the following day, animals were assigned to treatment conditions involving a single subcutaneous (sc) injection of paraben dissolved in 0.10 ml peanut oil in the nape. In Experiment 1, diestrous females and males received 0 (vehicle), 1, 3, or 9 mg BP (n=10 per dose). In Experiment 2, diestrous females and males received vehicle, 1, 3 or 9 mg PP (n=10 per dose). Sequential assignment within each experiment ensured that there was minimal overlap in treatment conditions among previously group-housed animals from the same cage. Table 1 provides BP and PP doses in mg/kg and animal weights in g for each treatment condition. At 30 min after injection, each animal was given a dietary supplement of 50 μg/kg ^{14}C-BPA in 0.2 g peanut butter in a glass dish placed inside its cage. Food, water, and bedding were removed to prevent contamination of the ^{14}C-BPA treatment. At 1 h after ^{14}C-BPA administration, each animal was anesthetized with isoflurane and blood was collected via cardiac puncture. Each animal was perfused with 20 ml phosphate-buffered saline (PBS) and tissues were collected in

pre-weighed scintillation vials. Tissue samples taken include the heart, lung, superficial adductor muscle from the hind leg, abdominal adipose, liver, and a cross-section of the kidney encompassing both the medulla and cortex. Female reproductive tissues taken include the whole uterus and both ovaries, whereas male reproductive tissues taken include one testis, one epididymis, one vesicular-coagulating (VC) gland, and one preputial gland. Vials were re-weighed following tissue collection to determine the sample wet mass.

In Experiment 3, mice were weighed and individually placed in a Plexiglas apparatus measuring 21×15×13 (l×w×h) cm with a wire-mesh grid floor. The cage was raised approximately 1 cm above a Teflon-coated stainless-steel surface covered with wax paper. Animals acclimated to the novel cages for 3 days prior to the start of the experiment. At the onset of darkness on the fourth day, diestrous females and males received a sc injection of vehicle, 3 mg BP (corresponding to 91.5 ± 5.2 mg/kg for females and 81.7 ± 2.6 mg/kg for males), or 3 mg PP (corresponding to 89.8 ± 4.3 mg/kg for females and 83.5 ± 4.1 mg/kg for males) dissolved in 0.10 ml peanut oil (n=10 per dose) in the nape. Urine was collected non-invasively at 2, 4, 6, 8, 10, and 12 h post-injection. All urine samples were placed into labeled vials and frozen at –20 °C at the time of collection.

Table 1 Mean (±SD) BP and PP doses in mg/kg and animal weights in g for each treatment condition. n = number of animals

		n	BPA Dose (µg/kg)	Paraben Dose (mg)	Paraben Dose (mg/kg)	Animal Weights (g)
BP	**Females**	10	50	vehicle	0.0 ± 0.0	28.7 ± 2.6
		10	50	1	35.0 ± 2.0	28.6 ± 1.7
		10	50	3	103.3 ± 9.4	29.2 ± 2.6
		10	50	9	310.0 ± 22.6	29.2 ± 2.2
	Males	10	50	vehicle	0.0 ± 0.0	39.1 ± 2.0
		10	50	1	26.9 ± 1.4	37.3 ± 1.9
		10	50	3	79.5 ± 5.5	37.9 ± 2.5
		10	50	9	242.1 ± 12.4	37.3 ± 1.9
PP	**Females**	10	50	vehicle	0.0 ± 0.0	26.5 ± 1.6
		10	50	1	35.5 ± 0.9	28.2 ± 0.7
		10	50	3	104.2 ± 6.1	28.9 ± 1.6
		10	50	9	323.7 ± 32.0	28.1 ± 3.0
	Males	10	50	vehicle	0.0 ± 0.0	39.3 ± 6.9
		10	50	1	25.0 ± 4.6	41.2 ± 7.4
		10	50	3	75.9 ± 12.6	40.5 ± 6.5
		10	50	9	239.0 ± 40.3	38.7 ± 7.2

2.4. Blood and tissue processing for liquid scintillation counting

Blood and tissue samples were processed for liquid scintillation counting following previously published procedures (deCatanzaro and Pollock, 2016; Pollock et al., 2016, 2014; Pollock and deCatanzaro, 2014). Blood samples were centrifuged at 1,500 *g* for 10 min and 10 µl serum was added to a scintillation vial containing 5 ml Ultima Gold. Tissue samples were solubilized by adding 1 ml SOLVABLE to each vial and placing vials in a 50 °C water bath for 4–5 h until completely dissolved. Following the addition of 5 ml Ultima Gold, vials were agitated to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) for 5 min to eliminate noise in the form of heat and luminescence. Radioactivity was then measured for 5 min per vial. The amount of radioactivity per sample, in disintegrations per minute (dpm), was calculated by the accompanying Quanta-Smart software package after correcting for sample coloration, luminescence, and static, as well as subtracting background levels of radiation. Frequent monitoring of all work surfaces and equipment ensured that contamination of samples did not occur. The final dpm measures were then normalized to the weight of the sample wet mass and reported as equivalent ng BPA/g tissue or ng BPA/ml serum.

2.5. Measurement of urinary E_2

Full procedures and validations for enzyme immunoassays for mouse urine were previously reported (Muir et al., 2001). Cross-reactivities for anti-E_2 are: E_2 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other

measured steroids <0.1%. Urinary E_2 levels were considered with and without correction for urinary creatinine, which adjusts for differential hydration and urinary concentration among animals, and reported as ng/mg creatinine and ng/ml urine, respectively.

2.6. Contamination control procedures

Ten diestrous females and ten males were injected sc with 0.10 ml peanut oil in the nape and administered the equivalent dose of ethanol in 0.2 g peanut butter. All other details, including anesthesia and perfusion, blood and tissue collection, sample processing, and scintillation counting were identical to those described above. The purpose of these negative control animals was to ensure that no external sources of radioactive contamination were present.

2.7. Statistical analyses

All statistical analyses were performed using the R software environment (R Core Team, 2016). A comparison-wise error rate of $\alpha < 0.05$ was employed for all statistical tests. Differences between ^{14}C-BPA treatments in animals given BP or PP were analyzed by univariate analysis of variance (ANOVA) for each tissue, with Holm-Bonferroni adjustments to correct for the number of tissues (Holm, 1979). Significant effects in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons. Differences between urinary E_2 concentrations were analyzed by factorial ANOVA comparing the effects of treatment and collection time-point (repeated measures). Significant main effects or interaction in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons of treatment at each collection time-point.

3. Results

3.1. Experiment 1: Measurement of ^{14}C-BPA in mice given BP

Radioactivity was measured in serum and tissues of diestrous females (Fig. 1) and males (Fig. 2) that received a sc injection of BP followed by a dietary supplement of ^{14}C-BPA. Concentrations of ^{14}C-BPA in the liver and kidney are reported in Table 2. Pre-treatment with BP induced a dose-dependent increase in serum and reproductive tissue concentrations of ^{14}C-BPA in both sexes. Comparisons were made among the four treatments for each of nine tissues in females. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the lung, $F(3,36)=5.15$, $p=0.028$; uterus, $F(3,36)=35.35$, $p<0.001$; ovaries, $F(3,36)=10.29$, $p<0.001$; kidney, $F(3,36)=4.85$, $p=0.031$; and serum, $F(3,36)=6.89$, $p=0.006$. Multiple comparisons revealed that the vehicle-treated group differed from the 1, 3, and 9 mg groups for the uterus. The vehicle-treated group also differed from the 9 mg group for the lung, ovaries, kidney, and serum. Comparisons were made among the four treatments for each of eleven tissues in males. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the testis, $F(3,36)=4.88$, $p=0.044$; epididymis, $F(3,36)=7.05$, p=0.007; and serum, $F(3,36)=7.82$, p=0.004. Multiple comparisons revealed that the vehicle-treated group differed from the 3 and 9 mg groups for the epididymis and serum, as well as the 9 mg group for the testis.

Fig. 1 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females following sc injection of vehicle, 1, 3, or 9 mg BP and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose). Difference from vehicle treatment in the same tissue: * $p < 0.05$; ** $p < 0.01$; + $p < 0.001$

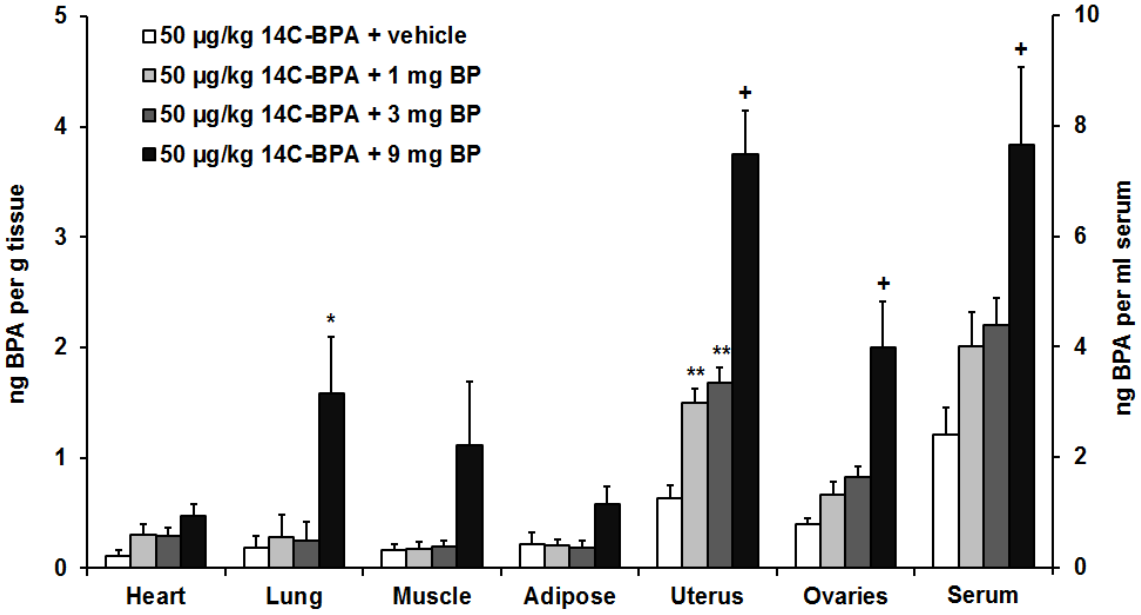


Fig. 2 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, testis, epididymis, VC gland, preputial gland, and serum of males following sc injection of vehicle, 1, 3, or 9 mg BP and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose). Difference from vehicle treatment in the same tissue: ** $p < 0.01$; + $p < 0.001$

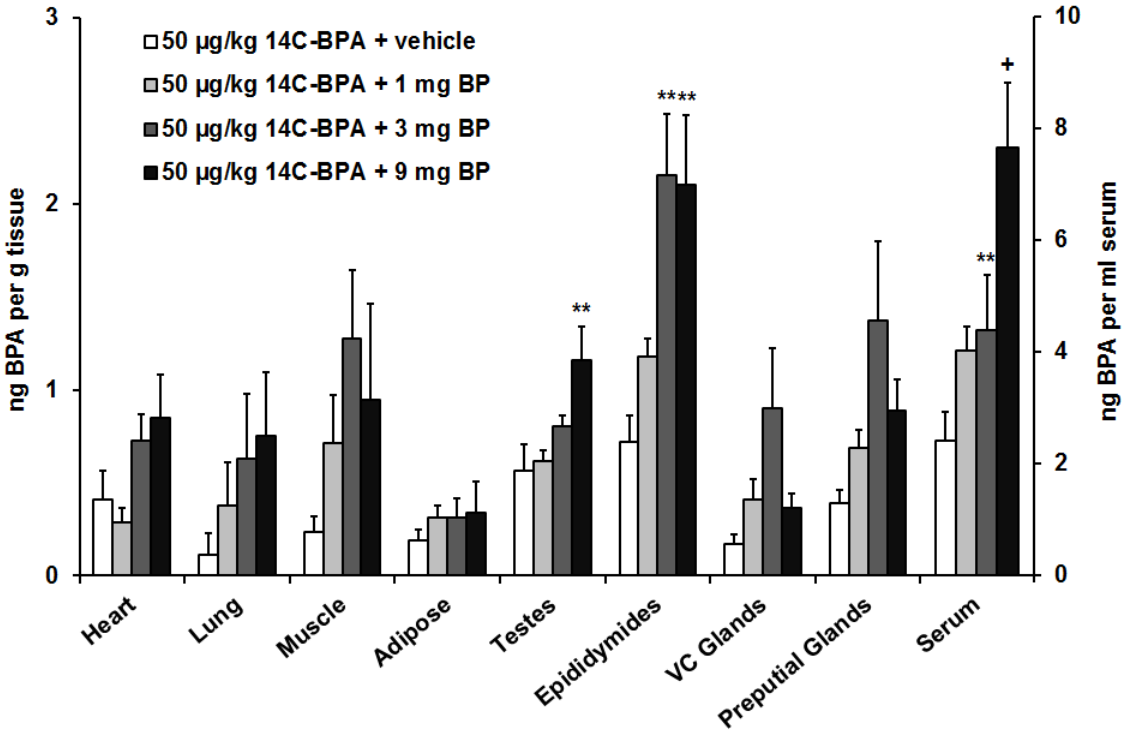


Table 2 Mean (±SE) concentration of ^{14}C-BPA in the liver and kidney of diestrous females and males following sc injection of BP or PP and subsequent dietary administration of 50 µg/kg ^{14}C-BPA. Difference from vehicle treatment in the same tissue: * $p < 0.05$

		Paraben Dose (mg)	Liver (ng BPA/g)	Kidney (ng BPA/g)
BP	**Females**	vehicle	16.7 ± 3.1	10.2 ± 2.0
		1	28.6 ± 3.6	11.2 ± 1.9
		3	25.9 ± 2.9	12.5 ± 1.5
		9	27.7 ± 3.6	25.2 ± 5.6*
	Males	vehicle	20.1 ± 2.5	33.1 ± 5.9
		1	24.9 ± 4.8	53.0 ± 7.2
		3	30.5 ± 6.5	50.7 ± 7.3
		9	40.0 ± 9.3	62.3 ± 14.6
PP	**Females**	vehicle	18.7 ± 1.6	12.4 ± 2.0
		1	22.2 ± 2.3	14.9 ± 1.5
		3	23.2 ± 3.0	10.1 ± 1.3
		9	24.7 ± 3.8	11.0 ± 1.5
	Males	vehicle	27.5 ± 4.0	52.6 ± 8.9
		1	28.0 ± 3.0	53.2 ± 6.3
		3	24.2 ± 2.4	48.4 ± 3.2
		9	44.4 ± 11.8	57.6 ± 7.9

3.2. Experiment 2: Measurement of ^{14}C-BPA in mice given PP

Radioactivity was measured in serum and tissues of diestrous females (Fig. 3) and males (Fig. 4) that received a sc injection of PP followed by a dietary supplement of ^{14}C-BPA. Concentrations of ^{14}C-BPA in the liver and kidney are reported in Table 2. Pre-treatment with PP selectively elevated ^{14}C-BPA concentrations in the uterus of females. Comparisons were made among the four treatments for each of nine tissues in females. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for uterus, $F(3,36)=2.55$, $p=0.006$. Multiple comparisons revealed that the vehicle-treated group differed from the 9 mg group for the uterus. Comparisons were made among the four treatments for each of eleven tissues in males. Minor elevations in ^{14}C-BPA concentrations were observed in certain tissues for the 9 mg PP group, such as the epididymides and serum; however, these effects were not statistically significant.

Fig. 3 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females following sc injection of vehicle, 1, 3, or 9 mg PP and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose). Difference from vehicle treatment in the same tissue: + $p < 0.001$

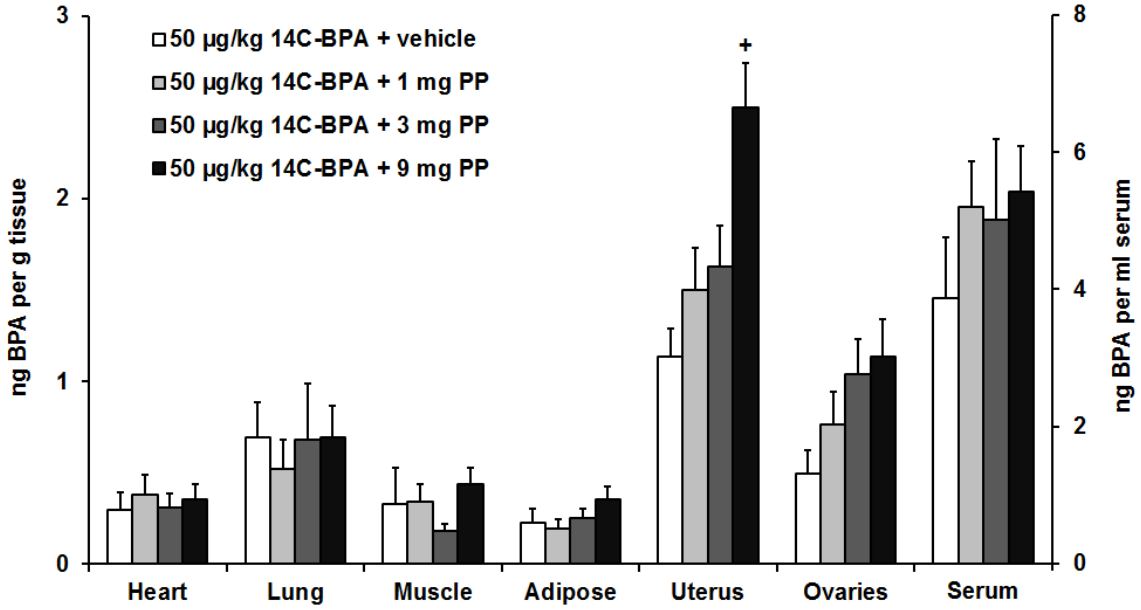
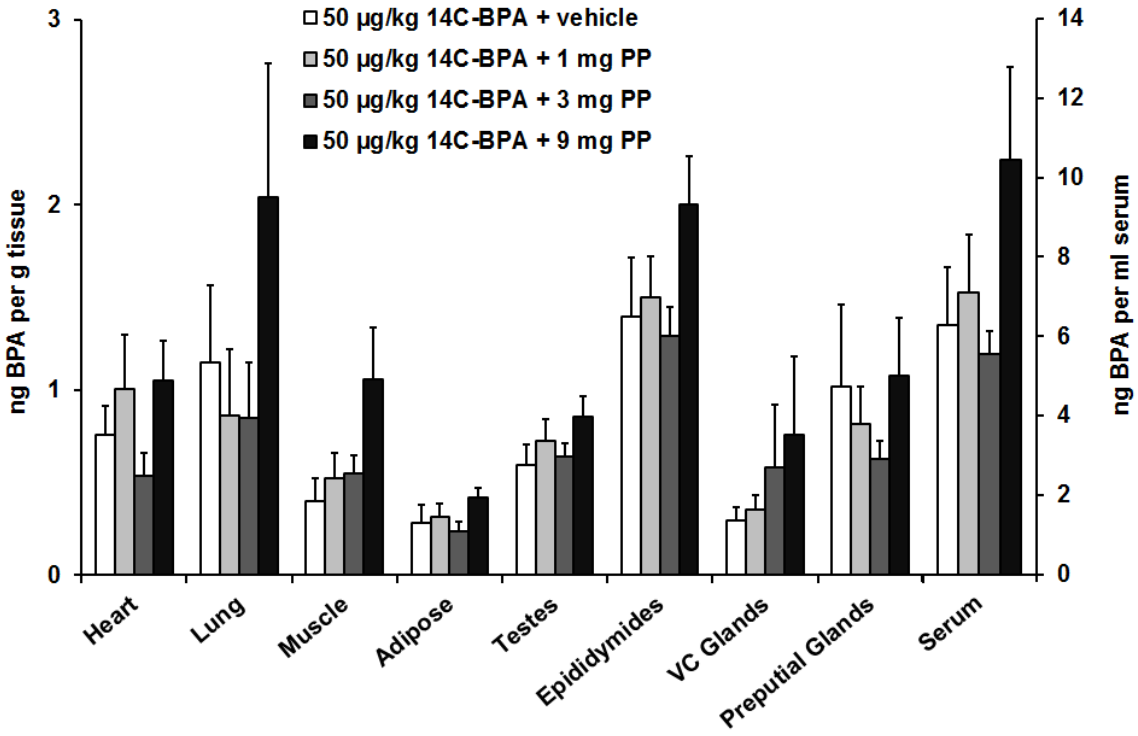


Fig. 4 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, testis, epididymis, VC gland, preputial gland, and serum of males following sc injection of vehicle, 1, 3, or 9 mg PP and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose).



3.3. Experiment 3: Measurement of urinary E_2 in mice given BP or PP

Urinary E_2 concentrations of diestrous females (Fig. 5) and males (Fig. 6) were measured after sc injection of vehicle, BP, or PP. Concentrations of E_2 are reported for uncorrected (ng E_2/ml urine) and corrected (ng E_2/mg creatinine) measures. In females, ANOVA on uncorrected measures showed a significant main effect of collection time-point, $F(5,135)=8.53$, $p<0.001$, and a significant interaction, $F(10,135)=3.00$, $p=0.002$. ANOVA on creatinine-corrected measures showed significant main effects of treatment, $F(2,27)=4.53$, $p=0.020$, and collection time-point, $F(5,135)=10.14$, $p<0.001$, and a significant interaction, $F(10,135)=4.22$, $p<0.001$. Multiple comparisons revealed that vehicle-treated females differed from BP-treated females at 6, 8, and 10 h after injection for both the uncorrected and corrected measures. In males, ANOVA on uncorrected measures showed a significant main effect of collection time-point, $F(5,105)=2.30$, $p=0.049$. ANOVA on creatinine-corrected measures also showed a significant main effect of collection time-point, $F(5,105)=2.72$, $p=0.024$. Multiple comparisons revealed that vehicle-treated males differed from BP-treated males at 8 h after injection for both the uncorrected and corrected measures.

Fig. 5 Mean (+SE) concentration of urinary E_2, expressed as ng E_2 / ml urine and ng E_2 / mg creatinine, following sc injection of vehicle, 3 mg BP, or 3 mg PP in diestrous females (n=10 per dose). Difference from vehicle treatment at the same time point: * $p < 0.05$; ** $p < 0.01$

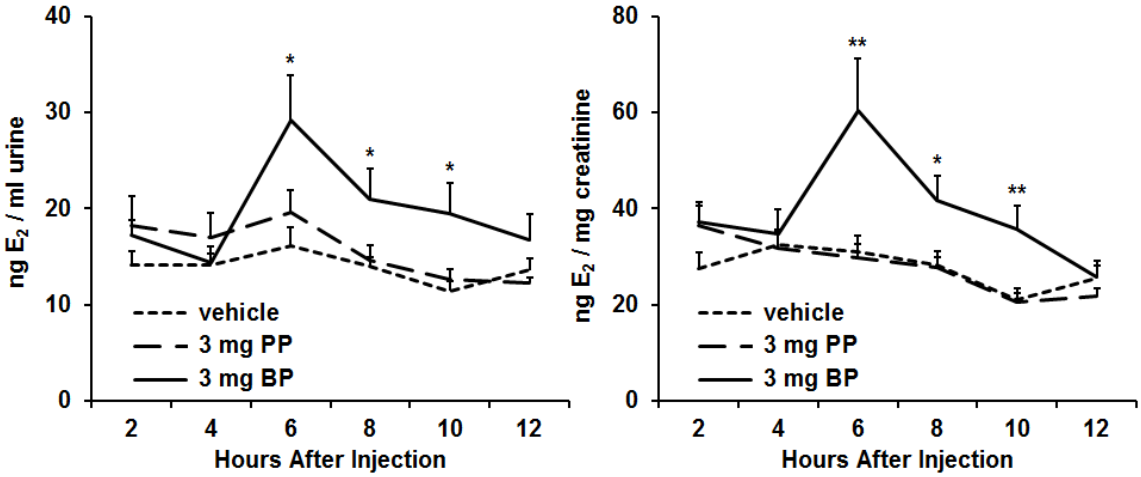
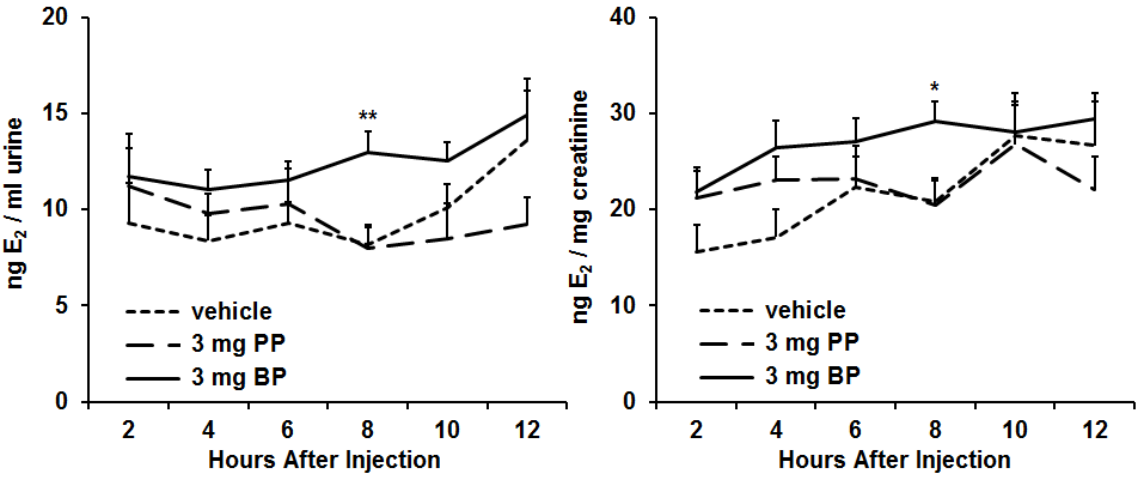


Fig. 6 Mean (+SE) concentration of urinary E_2, expressed as ng E_2 / ml urine and ng E_2 / mg creatinine, following sc injection of vehicle, 3 mg BP, or 3 mg PP in males (n=10 per dose). Difference from vehicle treatment at the same time point: * $p < 0.05$; ** $p < 0.01$



3.4. Radioactivity in contamination control animals

Ten diestrous females and ten males were exposed to the same experimental design as in Experiments 1 and 2, except that these negative control animals received a non-radioactive peanut butter supplement. Measures of radioactivity for these animals were at baseline for serum (0 ng BPA/ml) and all tissues (0 ng BPA/g), except the liver of two males (0.03 and 0.04 ng BPA equivalent/g), the liver of one female (0.10 ng BPA equivalent/g), and the kidney of another female (0.04 ng BPA equivalent/g). Measures of radioactivity in control animals were in a range entirely distinct from that of animals treated with ^{14}C-BPA, indicating no external sources of radioactive contamination.

4. Discussion

These data demonstrate *in vivo* modulation of the pharmacokinetics of BPA by PP and BP, as well as an effect of BP on excreted endogenous E_2. There are numerous potential mechanisms through which parabens, BPA, and E_2 could interact. These mechanisms include direct actions at ER, transport proteins in blood, or enzymes involved in steroid metabolism. Relative to E_2, BPA and BP show about 10,000-fold lower affinity *in vitro* for ERα, while PP shows about 30,000-fold lower affinity (Miller et al., 2001; Routledge et al., 1998). However, direct actions of BP or PP at ER would likely result in an opposite effect to that observed in our data, as these actions would be competitive with binding of ^{14}C-BPA. Indeed, pre-treatment with E_2 or the ER antagonist ICI 182,780 reduced ^{14}C-BPA concentrations in the uteri of rats (Pollock and deCatanzaro, 2014). Similarly, actions of BP or PP on transport proteins in blood would

likely be competitive with ^{14}C-BPA, thereby limiting its distribution and reducing ^{14}C-BPA concentrations in serum and tissues. Actions of BP and PP on metabolic enzymes are sufficient to explain the observed elevation of ^{14}C-BPA concentrations in serum and tissues and E_2 concentrations in urine. The major metabolite of BPA is the monoglucuronide conjugate produced via UGT (Kurebayashi et al., 2010; Zalko et al., 2003). Other metabolites formed in smaller quantities include the monosulfate conjugate produced via SULT and glucuronide/sulfate diconjugates (Inoue et al., 2016; Kurebayashi et al., 2010; Yalcin et al., 2016; Zalko et al., 2003). Urinary metabolite profiles of parabens vary, with MP being primarily sulfated (63%) and BP being primarily glucuronidated (87%) (Moos et al., 2016). Parabens are competitive inhibitors of SULT1A1 (Prusakiewicz et al., 2007) and numerous UGT isoforms, including UGT1A1, UGT1A8, UGT1A9, UGT2B7, UGT2B15, and UGT2B17 (Abbas et al., 2010). In addition to SULT and UGT, estrogen concentrations are regulated by CYP and hydroxysteroid dehydrogenase (HSD) (Dumas and Diorio, 2011). Whereas parabens were shown to be inactive towards 17β-HSD4 (NIH, 2010), they could competitively inhibit CYP1A1, CYP1A2, CYP2B, CYP3A, CYP2C, and CYP2E1 (Ozaki et al., 2016). Given the established inhibitory actions of parabens on UGT, SULT, and CYP, we suggest that these enzymes play critical roles in the mechanisms underlying our findings.

These data show that BP markedly elevates concentrations of ^{14}C-BPA in serum and select tissues, such as the lungs, uterus, ovaries, testes, and epididymides. The localization of ^{14}C-BPA is consistent with the profile of tissues with moderate to high ERα and ERβ expression (Kuiper et al., 1997). The impact of 1–9 mg BP on ^{14}C-BPA

concentrations is also consistent with that previously shown for 0.6–18 mg triclosan (Pollock et al., 2014). In contrast, pre-treatment with 9 mg PP only magnified concentrations of ^{14}C-BPA in the uterus. Measures of radioactivity reflect total BPA, including both conjugated and unconjugated species. Phase II metabolism of BPA occurs at one or both of the hydroxyl groups located on either end of the molecule (Zalko et al., 2003). Conjugation would not influence measures of radioactivity, as the ^{14}C-BPA used in these studies is labeled within the phenolic rings. We have previously shown that, following dietary administration of 50 µg/kg BPA, the majority of BPA in the uterus of female rats is unconjugated (Pollock and deCatanzaro, 2014). We administered ^{14}C-BPA in a dietary supplement, as ingestion of BPA from food, beverages, and indoor dust accounts for 85–95% of total exposure in adult humans (EFSA, 2015). Since the present study involves acute exposure, we did not quantify concentrations of ^{14}C-BPA in urine or feces. Elsewhere (Kurebayashi et al., 2005), only 1% of the total dose was present in urine of male and female rats during the initial 4 h after oral administration of 100 µg/kg ^{14}C-BPA. The majority of ^{14}C-BPA was located in the stomach, intestines, and liver during the first hour after oral administration (Kim et al., 2004). By 168 h after exposure, female rats had excreted 34% of the total dose in urine and 64% in feces, whereas male rats excreted 10% in urine and 88% in feces (Kurebayashi et al., 2005).

We also showed elevated concentrations of E_2 in urine following BP, but not PP, administration. This effect was most pronounced in females around 6–10 h after injection and in males around 8 h after injection. The impact of 3 mg BP on urinary E_2 in females is consistent with that previously shown for 1–2 mg triclosan (Pollock et al., 2016). The

discrepancy between effects of BP and PP on urinary E_2 is concordant with evidence that BP is a more potent inhibitor of SULT and CYP activity (Ozaki et al., 2016; Prusakiewicz et al., 2007). The discrepancy between males and females may be due to metabolic differences; male rodents excrete subcutaneously-injected BP more rapidly and completely than do female rodents (Aubert et al., 2012). Measures of urinary E_2 generally reflect systemic trends in estrogen activity (deCatanzaro et al., 2004, 2003; Muir et al., 2001; Thorpe et al., 2014, 2013). Whereas estrogen conjugates are predominant in circulation and act as a storage reservoir (Reed et al., 2005), unconjugated E_2 is primarily excreted in mouse urine (Muir et al., 2001). Unlike blood, urine can also be collected non-invasively at multiple time-points. Our demonstration that parabens can elevate endogenous E_2 is important because minor but persistent elevations in E_2 can contribute to adverse health and reproductive outcomes in mammals. Increased E_2 can disrupt intrauterine blastocyst implantation and lead to pregnancy failure (Thorpe et al., 2013). In humans, increased estrogen activity from hormone-replacement therapy correlates with increased risk of breast, endometrial, and ovarian cancers (Million Women Study Collaborators, 2007, 2005, 2003).

We administered BP and PP via sc injection, as the presence of parabens in cosmetics and personal care products leads to dermal absorption (El Hussein et al., 2007; Guo and Kannan, 2013). Aubert et al. (2012) found that sc injection produces more rapid systemic availability of parabens than does dermal absorption. They found maximum plasma concentrations of 614 and 1,033 ng/ml for BP and PP respectively 8 h after dermal administration of 100 mg/kg to female rats. In contrast, maximum plasma BP

concentration following sc injection was 12,190 ng/ml and occurred 4 h after exposure. In the current study, we examined a relatively short (30-min) latency between BP or PP and ^{14}C-BPA administration, which was also effective in a similar study of triclosan (Pollock et al., 2014). Skin permeability of parabens is inversely related to lipophilicity, with MP being the most permeable and parabens with longer side-chains, such as PP and BP, being less permeable (El Hussein et al., 2007; Pedersen et al., 2007). Following dermal administration of 100 mg/kg BP or PP to rats, around half of the total dose was unabsorbed and recovered from the application site (Aubert et al., 2012). Dermal metabolism in rodents may also contribute to lower uptake of parabens following topical application compared to sc injection (Harville et al., 2007; Prusakiewicz et al., 2006). However, rodent studies may not accurately reflect dermal absorption in humans, as esterase activity in rodent skin microsomes is 2–3 orders of magnitude greater than that in human skin microsomes (Harville et al., 2007; Prusakiewicz et al., 2006). We have only examined the capacity of parabens to alter the pharmacokinetics of BPA acutely via one route of exposure; future studies should explore repeated administrations and other exposure routes.

The doses of BP and PP administered in the present study are greater than typical exposure levels in the general public. Aggregate exposure estimates for MP, EP, PP, and BP from cosmetics and personal care products range from 1.26 to 4.13 mg/kg/day (Cowan-Ellsberry and Robison, 2009). However, exposure estimates rarely account for other sources of exposure, such as from foods, beverages, and pharmaceuticals (Błędzka et al., 2014). Published exposure estimates also may not accurately convey occupational

exposure levels. This is important considering the wide variance in urinary paraben concentrations, where the highest measured value of PP, for example, was over 900 times greater than the geometric mean (Calafat et al., 2010). Evidence of phase I and II metabolic enzyme inhibition has been demonstrated for numerous other environmental chemicals and their metabolites, including tetrabromobisphenol A (Gosavi et al., 2013; Harju et al., 2007; NIH, 2010), phthalates (Ozaki et al., 2016), polychlorinated biphenyls (Kester et al., 2000; Wang and James, 2007), and polyhalogenated aromatic hydrocarbons (Kester et al., 2002). Given the potential adverse health outcomes of persistently heightened estrogenic activity, these findings demonstrate the need to consider studies of multiple toxicants when determining regulatory exposure limits for endocrine-active chemicals.

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References

Abbas, S., Greige-Gerges, H., Karam, N., Piet, M.-H., Netter, P., Magdalou, J., 2010. Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man. Drug Metab. Pharmacokinet. 25, 568–577. doi:10.2133/dmpk.DMPK-10-RG-013

Andersen, F.A., 2008. Final amended report on the safety assessment of methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, and benzylparaben as used in cosmetic products. Int. J. Toxicol. 27, 1–82. doi:10.1080/10915810500257113

Aubert, N., Ameller, T., Legrand, J.J., 2012. Systemic exposure to parabens: Pharmacokinetics, tissue distribution, excretion balance and plasma metabolites of [^{14}C]-methyl-, propyl- and butylparaben in rats after oral, topical or subcutaneous administration. Food Chem. Toxicol. 50, 445–454. doi:10.1016/j.fct.2011.12.045

Berger, R.G., Foster, W.G., deCatanzaro, D., 2010. Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. Reprod. Toxicol. 30, 393–400. doi:10.1016/j.reprotox.2010.06.006

Berger, R.G., Hancock, T., deCatanzaro, D., 2007. Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. Reprod. Toxicol. 23, 138–144. doi:10.1016/j.reprotox.2006.09.005

Berger, R.G., Shaw, J., deCatanzaro, D., 2008. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone

and 17β-estradiol. Reprod. Toxicol. 26, 94–99. doi:10.1016/j.reprotox.2008.06.007

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. Toxicol. Sci. 54, 138–153. doi:10.1093/toxsci/54.1.138

Błędzka, D., Gromadzińska, J., Wąsowicz, W., 2014. Parabens. From environmental studies to human health. Environ. Int. 67, 27–42. doi:10.1016/j.envint.2014.02.007

Byers, S.L., Wiles, M. V., Dunn, S.L., Taft, R.A., 2012. Mouse estrous cycle identification tool and images. PLoS One 7, e35538. doi:10.1371/journal.pone.0035538

Byford, J.R., Shaw, L.E., Drew, M.G.B., Pope, G.S., Sauer, M.J., Darbre, P.D., 2002. Oestrogenic activity of parabens in MCF7 human breast cancer cells. J. Steroid Biochem. Mol. Biol. 80, 49–60. doi:10.1016/S0960-0760(01)00174-1

Calafat, A.M., Ye, X., Wong, L.Y., Bishop, A.M., Needham, L.L., 2010. Urinary concentrations of four parabens in the U.S. Population: NHANES 2005–2006. Environ. Health Perspect. 118, 679–685. doi:10.1289/ehp.0901560

CDC (Centers for Disease Control and Prevention), 2015. Fourth national report on human exposure to environmental chemicals. https://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Aug2014.pdf (accessed 11.10.16).

Charles, A.K., Darbre, P.D., 2013. Combinations of parabens at concentrations measured in human breast tissue can increase proliferation of MCF-7 human breast cancer

cells. J. Appl. Toxicol. 33, 390–398. doi:10.1002/jat.2850

Cowan-Ellsberry, C.E., Robison, S.H., 2009. Refining aggregate exposure: Example using parabens. Regul. Toxicol. Pharmacol. 55, 321–329. doi:10.1016/j.yrtph.2009.08.004

Crawford, B.R., deCatanzaro, D., 2012. Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A. Reprod. Toxicol. 34, 607–613. doi:10.1016/j.reprotox.2012.09.008

Darbre, P.D., Aljarrah, A., Miller, W.R., Coldham, N.G., Sauer, M.J., Pope, G.S., 2004. Concentrations of parabens in human breast tumours. J. Appl. Toxicol. 24, 5–13. doi:10.1002/jat.958

deCatanzaro, D., Muir, C., Beaton, E.A., Jetha, M., 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69, 687–696. doi:10.1016/j.steroids.2004.07.002

deCatanzaro, D., Muir, C., Beaton, E., Jetha, M., Nadella, K., 2003. Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 126, 407–414. doi:10.1530/reprod/126.3.407

deCatanzaro, D., Pollock, T., 2016. Absorption and distribution of estradiol from male seminal emissions during mating. J. Endocrinol. 231, 245–257. doi:10.1530/JOE-16-0247

Dumas, I., Diorio, C., 2011. Estrogen pathway polymorphisms and mammographic

density. Anticancer Res. 31, 4369–4386.

EFSA (European Food Safety Authority), 2015. Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA J. 13, 3978. doi:10.2903/j.efsa.2015.3978

El Hussein, S., Muret, P., Berard, M., Makki, S., Humbert, P., 2007. Assessment of principal parabens used in cosmetics after their passage through human epidermis-dermis layers (*ex-vivo* study). Exp. Dermatol. 16, 830–836. doi:10.1111/j.1600-0625.2007.00625.x

Gomez, E., Pillon, A., Fenet, H., Rosain, D., Duchesne, M.J., Nicolas, J.C., Balaguer, P., Casellas, C., 2005. Estrogenic activity of cosmetic components in reporter cell lines: parabens, UV screens, and musks. J. Toxicol. Environ. Health. A 68, 239–251. doi:10.1080/15287390590895054

Gosavi, R.A., Knudsen, G.A., Birnbaum, L.S., Pedersen, L.C., 2013. Mimicking of estradiol binding by flame retardants and their metabolites: A crystallographic analysis. Environ. Health Perspect. 121, 1194–1199. doi:10.1289/ehp.1306902

Guerra, M.T., Furlong, H.C., Kempinas, W.G., Foster, W.G., 2016. Effects of *in vitro* exposure to butylparaben and di-(2 ethylhexyl) phthalate, alone or in combination, on ovarian function. J. Appl. Toxicol. 36, 1235–1245. doi:10.1002/jat.3335

Guo, Y., Kannan, K., 2013. A survey of phthalates and parabens in personal care products from the United States and its implications for human exposure. Environ. Sci. Technol. 47, 14442–14449. doi:10.1021/es4042034

Harju, M., Hamers, T., Kamstra, J.H., Sonneveld, E., Boon, J.P., Tysklind, M.,

Andersson, P.L., 2007. Quantitative structure–activity relationship modeling on *in vitro* endocrine effects and metabolic stability involving 26 selected brominated flame retardants. Environ. Toxicol. Chem. 26, 816–826. doi:10.1897/06-308R.1

Harville, H.M., Voorman, R., Prusakiewicz, J.J., 2007. Comparison of paraben stability in human and rat skin. Drug Metab. Lett. 1, 17–21. doi:10.2174/187231207779814300

Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. 6, 65–70. doi:10.2307/4615733

Inoue, H., Kemanai, S., Sano, C., Kato, S., Yokota, H., Iwano, H., 2016. Bisphenol A glucuronide/sulfate diconjugate in perfused liver of rats. J. Vet. Med. Sci. 78, 733–737. doi:10.1292/jvms.15-0573

James, M.O., Ambadapadi, S., Falany, C., 2015. Triclosan inhibits the activity of expressed human sulfotransferases (SULTs) towards their diagnostic substrates. FASEB J. 29, 622 (Abstract).

James, M.O., Li, W., Summerlot, D.P., Rowland-Faux, L., Wood, C.E., 2010. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ. Int. 36, 942–949. doi:10.1016/j.envint.2009.02.004

Kang, H.S., Kyung, M.S., Ko, A., Park, J.H., Hwang, M.S., Kwon, J.E., Suh, J.H., Lee, H.S., Moon, G.I., Hong, J.H., Hwang, I.G., 2016. Urinary concentrations of parabens and their association with demographic factors: A population-based cross-sectional study. Environ. Res. 146, 245–251. doi:10.1016/j.envres.2015.12.032

Kester, M.H.A., Bulduk, S., Tibboel, D., Meinl, W., Glatt, H., Falany, C.N., Coughtrie,

M.W.H., Bergman, A., Safe, S.H., Kuiper, G.G.J.M., Schuur, A.G., Brouwer, A., Visser, T.J., 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: A novel pathway explaining the estrogenic activity of PCBs. Endocrinology 141, 1897–1900. doi:10.1210/endo.141.5.7530

Kester, M.H.A., Bulduk, S., van Toor, H., Tibboel, D., Meinl, W., Glatt, H., Falany, C.N., Coughtrie, M.W.H., Schuur, A.G., Brouwer, A., Visser, T.J., 2002. Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J. Clin. Endocrinol. Metab. 87, 1142–1150. doi:10.1210/jcem.87.3.8311

Kim, C.S., Sapienza, P.P., Ross, I.A., Johnson, W., Luu, H.M.D., Hutter, J.C., 2004. Distribution of bisphenol A in the neuroendocrine organs of female rats. Toxicol. Ind. Health 20, 41–50. doi:10.1191/0748233704th186oa

Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., Gustafsson, J.A., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138, 863–870.

Kurebayashi, H., Nagatsuka, S.I., Nemoto, H., Noguchi, H., Ohno, Y., 2005. Disposition of low doses of ^{14}C-bisphenol A in male, female, pregnant, fetal, and neonatal rats. Arch. Toxicol. 79, 243–252. doi:10.1007/s00204-004-0628-2

Kurebayashi, H., Okudaira, K., Ohno, Y., 2010. Species difference of metabolic clearance of bisphenol A using cryopreserved hepatocytes from rats, monkeys and humans. Toxicol. Lett. 198, 210–215. doi:10.1016/j.toxlet.2010.06.017

Lemini, C., Hernández, a, Jaimez, R., Franco, Y., Avila, M.E., Castell, a, 2004. Morphometric analysis of mice uteri treated with the preservatives methyl, ethyl, propyl, and butylparaben. Toxicol. Ind. Health 20, 123–132. doi:10.1191/0748233704th202oa

Miller, D., Wheals, B.B., Beresford, N., Sumpter, J.P., 2001. Estrogenic activity of phenolic additives determined by an *in vitro* yeast bioassay. Environ. Health Perspect. 109, 133–138.

Million Women Study Collaborators, 2007. Ovarian cancer and hormone replacement therapy in the Million Women Study. Lancet 369, 1703–1710. doi:10.1016/S0140-6736(07)60534-0

Million Women Study Collaborators, 2005. Endometrial cancer and hormone-replacement therapy in the Million Women Study. Lancet 365, 1543–1551. doi:10.1016/S0140-6736(05)66455-0

Million Women Study Collaborators, 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 362, 419–427. doi:10.1016/S0140-6736(03)14065-2

Moos, R.K., Angerer, J., Dierkes, G., Brüning, T., Koch, H.M., 2016. Metabolism and elimination of methyl, *iso*- and *n*-butyl paraben in human urine after single oral dosage. Arch. Toxicol. 90, 2699–2709. doi:10.1007/s00204-015-1636-0

Muir, C., Spironello-Vella, E., Pisani, N., deCatanzaro, D., 2001. Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Horm. Metab. Res. 33, 653–658. doi:10.1055/s-2001-

18692

NIH (National Institutes of Health, National Center for Biotechnology Information), 2010. PubChem BioAssay Database; AID=893. https://pubchem.ncbi.nlm.nih.gov/bioassay/893 (accessed 1.14.17).

Oishi, S., 2002a. Effects of butyl paraben on the male reproductive system in mice. Arch. Toxicol. 76, 423–429. doi:10.1007/s00204-002-0360-8

Oishi, S., 2002b. Effects of propyl paraben on the male reproductive system. Food Chem. Toxicol. 40, 1807–1813. doi:10.1016/S0278-6915(02)00204-1

Ozaki, H., Sugihara, K., Watanabe, Y., Ohta, S., Kitamura, S., 2016. Cytochrome P450-inhibitory activity of parabens and phthalates used in consumer products. J. Toxicol. Sci. 41, 551–560.

Pedersen, S., Marra, F., Nicoli, S., Santi, P., 2007. *In vitro* skin permeation and retention of parabens from cosmetic formulations. Int. J. Cosmet. Sci. 29, 361–367. doi:10.1111/j.1468-2494.2007.00388.x

Pollock, T., deCatanzaro, D., 2014. Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses. Reprod. Toxicol. 49, 145–154. doi:10.1016/j.reprotox.2014.08.005

Pollock, T., Greville, L.J., Tang, B., deCatanzaro, D., 2016. Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice. Reprod. Toxicol. 65, 394–401. doi:10.1016/j.reprotox.2016.09.004

Pollock, T., Tang, B., deCatanzaro, D., 2014. Triclosan exacerbates the presence of ^{14}C-bisphenol A in tissues of female and male mice. Toxicol. Appl. Pharmacol. 278,

116–123. doi:10.1016/j.taap.2014.04.017

Prusakiewicz, J.J., Ackermann, C., Voorman, R., 2006. Comparison of skin esterase activities from different species. Pharm. Res. 23, 1517–1524. doi:10.1007/s11095-006-0273-y

Prusakiewicz, J.J., Harville, H.M., Zhang, Y., Ackermann, C., Voorman, R.L., 2007. Parabens inhibit human skin estrogen sulfotransferase activity: Possible link to paraben estrogenic effects. Toxicology 232, 248–256. doi:10.1016/j.tox.2007.01.010

R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing. https://www.r-project.org (accessed 11.10.16).

Reed, M.J., Purohit, A., Woo, L.W.L., Newman, S.P., Potter, B.V.L., 2005. Steroid sulfatase: Molecular biology, regulation, and inhibition. Endocr. Rev. 26, 171–202. doi:10.1210/er.2004-0003

Rochester, J.R., 2013. Bisphenol A and human health: A review of the literature. Reprod. Toxicol. 42, 132–155. doi:10.1016/j.reprotox.2013.08.008

Routledge, E.J., Parker, J., Odum, J., Ashby, J., Sumpter, J.P., 1998. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicol. Appl. Pharmacol. 153, 12–9. doi:10.1006/taap.1998.8544

SCCP, 2008. Scientific Committee on Consumer Products Opinion on: Parabens COLIPA no. P82. https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_138.pdf

(accessed 11.10.16).

SCCS, 2013. Scientific Committee on Consumer Safety Opinion on: Parabens COLIPA no. P82. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_132.pdf (accessed 11.10.16).

Seachrist, D.D., Bonk, K.W., Ho, S.M., Prins, G.S., Soto, A.M., Keri, R.A., 2016. A review of the carcinogenic potential of bisphenol A. Reprod. Toxicol. 59, 167–182. doi:10.1016/j.reprotox.2015.09.006

Smith, K.W., Braun, J.M., Williams, P.L., Ehrlich, S., Correia, K.F., Calafat, A.M., Ye, X., Ford, J., Keller, M., Meeker, J.D., Hauser, R., 2012. Predictors and variability of urinary paraben concentrations in men and women, including before and during pregnancy. Environ. Health Perspect. 120, 1538–1543. doi:10.1289/ehp.1104614

Soni, M.G., Carabin, I.G., Burdock, G.A., 2005. Safety assessment of esters of *p*-hydroxybenzoic acid (parabens). Food Chem. Toxicol. 43, 985–1015. doi:10.1016/j.fct.2005.01.020

Thorpe, J.B., Burgess, P.S., Sadkowski, M., deCatanzaro, D., 2013. Estrogen-progesterone balance in the context of blastocyst implantation failure induced by predator stress. Psychoneuroendocrinology 38, 3048–3056. doi:10.1016/j.psyneuen.2013.09.001

Thorpe, J.B., Gould, K.E., Borman, E.D., deCatanzaro, D., 2014. Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*). Horm. Metab. Res. 46, 211–218. doi:10.1055/s-0033-

1363958

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. Pharmacology 32, 1162–1169. doi:10.1124/dmd.104.000273.the

Wang, L.-Q., James, M.O., 2007. Sulfonation of 17β-estradiol and inhibition of sulfotransferase activity by polychlorobiphenylols and celecoxib in channel catfish, *Ictalurus punctatus*. Aquat. Toxicol. 81, 286–292. doi:10.1016/j.aquatox.2006.12.011

Watanabe, Y., Kojima, H., Takeuchi, S., Uramaru, N., Ohta, S., Kitamura, S., 2013. Comparative study on transcriptional activity of 17 parabens mediated by estrogen receptor α and β and androgen receptor. Food Chem. Toxicol. 57, 227–234. doi:10.1016/j.fct.2013.03.036

Yalcin, E.B., Kulkarni, S.R., Slitt, A.L., King, R., 2016. Bisphenol A sulfonation is impaired in metabolic and liver disease. Toxicol. Appl. Pharmacol. 292, 75–84. doi:10.1016/j.taap.2015.12.009

Zalko, D., Soto, A.M., Dolo, L., Dorio, C., Rathahao, E., Debrauwer, L., Faure, R., Cravedi, J.P., 2003. Biotransformations of bisphenol A in a mammalian model: Answers and new questions raised by low-dose metabolic fate studies in pregnant CD1 mice. Environ. Health Perspect. 111, 309–319. doi:10.1289/ehp.5603

Zhang, L., Ding, S., Qiao, P., Dong, L., Yu, M., Wang, C., Zhang, M., Zhang, L., Li, Y., Tang, N., Chang, B., 2016. *n*-butylparaben induces male reproductive disorders via

regulation of estradiol and estrogen receptors. J. Appl. Toxicol. 36, 1223–1234. doi:10.1002/jat.3291

Ziv-Gal, A., Flaws, J.A., 2016. Evidence for bisphenol A-induced female infertility: A review (2007–2016). Fertil. Steril. 106, 827–856. doi:10.1016/j.fertnstert.2016.06.027

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Chapter 6

Influence of tetrabromobisphenol A, with or without concurrent triclosan, upon bisphenol A and estradiol concentrations in mice

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Authors' Contributions

Tyler Pollock: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

Leanna Mantella: Assistance with data collection.

Vanessa Reali: Assistance with data collection.

Denys deCatanzaro: Assistance with experimental design, manuscript editing and preparation.

Abstract

Background: Humans are commonly exposed to multiple environmental chemicals, including tetrabromobisphenol A (TBBPA; a flame retardant), triclosan (an antimicrobial agent), and bisphenol A (BPA; polycarbonate plastics). These chemicals are readily absorbed and potentially interact.

Objectives: We sought to determine whether TBBPA, given alone or in combination with triclosan, can modulate the concentrations of BPA and 17β-estradiol (E2).

Methods: Female and male CF1 mice were each given a subcutaneous injection of 0–27 mg TBBPA, with or without concurrent 0.33 mg triclosan, followed by dietary administration of 50 µg/kg bodyweight ^{14}C-BPA. Radioactivity was measured in blood serum and tissues through liquid scintillation counting. In subsequent experiments, female and male CF1 mice were each given a subcutaneous injection of 0 or 1 mg TBBPA, and E2 was measured in urine 2–12 h after injection.

Results: Doses as low as 1 mg TBBPA significantly elevated ^{14}C-BPA concentrations in the uterus and ovaries of females; the testes, epididymides, vesicular-coagulating glands, and preputial glands of males; and blood serum, heart, lungs, and kidneys of both sexes. They also elevated urinary E2 concentrations. Lower doses of TBBPA or triclosan that had no effect on their own elevated ^{14}C-BPA concentrations when the two substances were given concurrently.

Conclusions: These data indicate that TBBPA, triclosan, and BPA interact *in vivo*, consistent with evidence that TBBPA and triclosan inhibit enzymes that are critical for BPA and E2 metabolism.

Introduction

Tetrabromobisphenol A (TBBPA; CAS 79-94-7) is the most-produced flame retardant, with global use over 170,000 tons/year (Health Canada 2013). Approximately 80% of TBBPA is used in reactive applications, where it is covalently bound to the polymer of epoxy resins for printed circuit boards in electronics equipment (Colnot et al. 2014; Shaw et al. 2014). The remaining 20% of TBBPA is used in additive applications, where it is physically blended with rather than chemically bound to the polymer, as in plastic housing for electronics equipment (Colnot et al. 2014; Shaw et al. 2014). Both reactive- and additive-treated products release TBBPA into the environment (Malkoske et al. 2016; Shaw et al. 2014). TBBPA has been detected in soil and sediment (Lee et al. 2015; Wang et al. 2015a; Zhu et al. 2014), surface and waste water (Kim et al. 2016; Xiong et al. 2015), and air and indoor dust (La Guardia and Hale 2015; Ni and Zeng 2013; Wang et al. 2015b; Wu et al. 2016b). Non-occupational TBBPA exposure in humans occurs via inhalation and ingestion of dust, as well as dermal contact with dust and free (unreacted) TBBPA in consumer products (Abdallah 2016; Knudsen et al. 2015). TBBPA is bioavailable in humans, as evidenced by its detection in human serum (Cariou et al. 2008; Fujii et al. 2014), plasma (Ho et al. 2017), breast milk (Abdallah and Harrad 2011; Fujii et al. 2014; Nakao et al. 2015), and adipose tissue (Cariou et al. 2008; Johnson-Restrepo et al. 2008).

The potential for TBBPA to act as an endocrine disrupting chemical is not well understood. Mechanisms of endocrine disruption by TBBPA could include actions on estrogen, androgen, glucocorticoid, and/or thyroid hormone receptors (Beck et al. 2016;

Hamers et al. 2006; Huang et al. 2013). Considering evidence of estrogenic actions, some studies found that TBBPA binds to estrogen receptor (ER) α in *in vitro* assays (Li et al. 2010; Olsen et al. 2003; Suzuki et al. 2013), whereas other studies found that TBBPA failed to bind ERα in *in vitro* assays (Dorosh et al. 2011; Hamers et al. 2006; Lee et al. 2012; Meerts et al. 2001; Miller et al. 2001; Molina-Molina et al. 2013; Riu et al. 2011a, 2011b) and molecular modeling (Zhuang et al. 2014). More recent work has examined indirect mechanisms of estrogenicity whereby TBBPA disrupts estrogen homeostasis (Honkisz and Wójtowicz 2015; Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016). One proposed mechanism is that TBBPA inhibits the metabolism of 17β-estradiol (E2), thus increasing its bioavailability, via interactions with conjugating enzymes (Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016). These enzymes include estrogen sulfotransferase (SULT), UDP-glucuronosyltransferase (UGT), cytochrome p450 (CYP), and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Dumas and Diorio 2011; Wikoff et al. 2016). Another proposed mechanism is that TBBPA enhances E2 secretion via actions on aromatase (CYP19) expression (Honkisz and Wójtowicz 2015).

We previously demonstrated *in vivo* interactions among bisphenol A (BPA), E2, and triclosan (CAS 3380-34-5), an antimicrobial agent found in soaps and cosmetics. Compared to vehicle-treated animals, male and female mice given a single dose of 0.6–18 mg triclosan showed greater concentrations of ^{14}C-BPA in blood serum and reproductive and other tissues (Pollock et al. 2014). Similarly, female mice given a single dose of 1–2 mg triclosan showed greater concentrations of exogenous ^{3}H-E2 in the uterus and natural E2 in urine than did vehicle-treated animals (Pollock et al. 2016). Blastocyst

implantation in inseminated female mice can be disrupted by high doses of BPA (Berger et al. 2007, 2008, 2010; Borman et al. 2015) and triclosan (Crawford and deCatanzaro 2012); lower doses of each substance that were insufficient on their own disrupted implantation when combined (Crawford and deCatanzaro 2012). Whereas triclosan alone was ineffective in a uterotrophic assay of weanling rats, elevated uterine weight occurred following concurrent triclosan and ethinyl estradiol exposure (Stoker et al. 2010). These findings are consistent with evidence that triclosan is conjugated by SULT, UGT, and CYP (Wu et al. 2016a) and can inhibit the activity of SULT and UGT toward other substances, including BPA and E2 (James et al. 2010, 2015; Wang et al. 2004).

As humans are routinely exposed to multiple potential endocrine-disrupting chemicals, it is important to investigate these chemicals' capacity to interact with each other and endogenous steroids *in vivo*. Here we undertook to measure the interactions of TBBPA, triclosan, and BPA. Whereas evidence of direct ER activation of TBBPA and triclosan is weak, BPA is a more established environmental estrogen (Rochester 2013; Seachrist et al. 2016; Ziv-Gal and Flaws 2016). Based on the proposed disruption of estrogen homeostasis via inhibitory actions of TBBPA on conjugating enzyme activity (Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016), we hypothesized that TBBPA would elevate BPA concentrations in female and male mice, and that this effect would be greatest in serum and estrogen-binding reproductive tissues. We hypothesized that actions of TBBPA would be additive with those of triclosan, consistent with evidence that triclosan also inhibits activity of conjugating enzymes (James et al. 2010; Wang et al. 2004). We tested these hypotheses by comparing the impact of TBBPA injection, either

alone or in combination with triclosan, on concentrations of ^{14}C-BPA in serum and tissues. We also hypothesized that TBBPA could elevate endogenous levels of E2, the most potent natural estrogen (Kuiper et al. 1997), testing this by measuring the impact of TBBPA injection on urinary E2.

Methods

Animals and housing

Female (30.5 ± 2.5 g) and male (40.2 ± 3.6 g) CF-1 mice aged 3–4 months were obtained from Charles River (Kingston, NY). In order to standardize timing within the cycle at an easily detected point where estrogen levels are moderate and relatively stable (Miller and Takahashi 2014), we selected diestrous females for use in experiments. These females were identified from a colony of mice with regular estrous cycles by vaginal cytology using published procedures (Byers et al. 2012). Animals were housed in polypropylene cages measuring 28×16×11 (l×w×h) cm with *ad libitum* access to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. All animals were treated humanely and with regard for alleviation of suffering. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University (Protocol 14-02-03).

Chemicals and materials

Triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol, ≥97% purity), 3,3′,5,5′-TBBPA (4,4′-isopropylidenebis[2,6-dibromophenol], ≥97% purity), 17β-E2 (≥98% purity), and creatinine standards were obtained from Sigma-Aldrich, St. Louis, MO. ^{14}C-BPA ([ring-[^{14}C](U)]-BPA, in ethanol, 0.1 mCi/mL, 106 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, 8 mL midi-vial scintillation vials were obtained from PerkinElmer, Waltham, MA. E2 antibodies and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA.

Experimental design and dosing

This research followed previously published procedures in this laboratory (Pollock et al. 2014, 2016). In brief, mice were weighed, individually housed, and each given a dietary supplement of 1 g peanut butter. About 14–16 h later at the onset of darkness on the following day, animals were randomly assigned to treatment conditions involving a single subcutaneous (sc) injection of TBBPA and/or triclosan dissolved in 0.05 mL peanut oil. In Experiment 1, diestrous females and males received vehicle, 1, 3, 9, or 27 mg TBBPA (n = 7 per dose). In Experiment 2, diestrous females (n = 7 per dose) and males (n = 6 per dose) received a single sc injection of vehicle, 0.33 mg TBBPA, 0.33 mg triclosan, or 0.33 mg TBBPA + 0.33 mg triclosan. Table 1 provides TBBPA and triclosan doses in mg/kg for each treatment condition. At 30 min after injection, each animal was given a dietary supplement of 50 µg/kg ^{14}C-BPA in 0.2 g peanut butter. Food, water, and bedding were removed to prevent contamination of the ^{14}C-BPA treatment. At

1 h after ^{14}C-BPA administration, each animal was anesthetized with isoflurane and blood was collected via cardiac puncture. Each animal was perfused with 20 mL phosphate-buffered saline (PBS) and tissues were collected in pre-weighed scintillation vials. Tissue samples taken include the heart, lung, superficial adductor muscle from the hind leg, abdominal adipose, liver, and a cross-section of the kidney encompassing both the medulla and cortex. Male reproductive tissues taken include one testis, one epididymis, one vesicular-coagulating (VC) gland, and one preputial gland. Female reproductive tissues taken include the whole uterus and both ovaries. Vials were re-weighed following tissue collection to determine the sample wet mass; no significant changes in tissue weights were observed (data not shown).

In Experiment 3, mice were weighed and individually placed in a Plexiglas apparatus measuring 21×15×13 (l×w×h) cm with a wire-mesh grid floor raised approximately 1 cm above a Teflon-coated stainless-steel surface covered with wax paper. Animals acclimated to the novel cages for 3 days prior to the start of the experiment. At the onset of darkness on the fourth day, diestrous females and males received a sc injection of vehicle or 1 mg TBBPA (corresponding to 33.8 ± 3.7 mg TBBPA/kg for females and 23.4 ± 2.4 mg TBBPA/kg for males) dissolved in 0.05 mL peanut oil (n=15 per dose). Urine was collected non-invasively at 2, 4, 6, 8, 10, and 12 h post-injection. All urine samples were placed into labeled vials and frozen at –20 °C at the time of collection.

Table 1 Mean (±SD) TBBPA and triclosan doses in mg/kg for each treatment condition.

n = number of animals

	n	BPA Dose (µg/kg)	TBBPA Dose (mg)	TBBPA Dose (mg/kg)	Triclosan Dose (mg)	Triclosan Dose (mg/kg)
Experiment 1						
Females	7	50	0	0.0 ± 0.0		
	7	50	1	32.7 ± 2.2		
	7	50	3	95.5 ± 4.8		
	7	50	9	291.3 ± 22.2		
	7	50	27	860.8 ± 67.2		
Males	7	50	0	0.0 ± 0.0		
	7	50	1	24.6 ± 1.4		
	7	50	3	76.7 ± 1.9		
	7	50	9	221.5 ± 9.6		
	7	50	27	686.4 ± 26.5		
Experiment 2						
Females	7	50	0	0.0 ± 0.0	0	0.0 ± 0.0
	7	50	0	0.0 ± 0.0	0.33	11.4 ± 0.6
	7	50	0.33	10.9 ± 0.6	0	0.0 ± 0.0
	7	50	0.33	10.8 ± 0.8	0.33	10.8 ± 0.8
Males	6	50	0	0.0 ± 0.0	0	0.0 ± 0.0
	6	50	0	0.0 ± 0.0	0.33	8.4 ± 0.6
	6	50	0.33	9.6 ± 0.5	0	0.0 ± 0.0
	6	50	0.33	8.0 ± 0.5	0.33	8.0 ± 0.5

We administered triclosan and TBBPA via sc injection to mimic dermal absorption of triclosan from personal care products (Fang et al. 2014; Queckenberg et al. 2010) and free (unreacted) TBBPA from dust and consumer products (Abdallah 2016; Knudsen et al. 2015). However, percutaneous penetration is incomplete compared to sc injection; up to 70% of dermally applied TBBPA is absorbed through rat skin (Knudsen et al. 2015) and up to 85% of dermally applied triclosan is absorbed through mouse skin (Fang et al. 2014). We administered ^{14}C-BPA in a dietary supplement to mimic ingestion of BPA from dust, food, and beverages, which accounts for approximately 85–95% of total exposure in adults (EFSA 2015). Dietary BPA exposure leads to less efficient first-pass hepatic metabolism and higher serum BPA concentrations than oral bolus (Sieli et al. 2011). The 30-min latency between TBBPA and ^{14}C-BPA administration and the 1-h latency between ^{14}C-BPA treatment and tissue collection were chosen based on an effective paradigm used in previous studies (Pollock et al. 2014, 2016). We selected 1–27 mg of TBBPA in Experiment 1 to establish the impact of a wide range of TBBPA exposures on tissue concentrations of ^{14}C-BPA. To investigate potential additive effects of TBBPA and triclosan in Experiment 2, we selected 0.33 mg such that the quantity of either substance was below the lowest effective dose of triclosan (0.6 mg) required to elevate ^{14}C-BPA concentrations (Pollock et al. 2014). When both substances were given concurrently, the combined quantity (0.66 mg) was greater than the lowest effective dose of triclosan used previously (Pollock et al. 2014). We selected 1 mg TBBPA for Experiment 3 as this dose was sufficient to modulate ^{14}C-BPA concentrations in Experiment 1. We measured urinary E2 as there are very low concentrations of estrogen

conjugates in mouse urine (Muir et al. 2001), whereas unconjugated E2 is abundant in urine and reflects systemic trends (deCatanzaro et al. 2003, 2004; Muir et al. 2001; Thorpe et al. 2013, 2014).

Blood and tissue processing for liquid scintillation counting

Blood and tissue samples were processed for liquid scintillation counting following previously published procedures (deCatanzaro and Pollock 2016; Pollock et al. 2014, 2016; Pollock and deCatanzaro 2014). Blood samples were centrifuged at 1,500 *g* for 10 min and 10 µL serum was added to a scintillation vial containing 5 mL Ultima Gold. Tissue samples were solubilized by adding 1 mL SOLVABLE to each vial and placing vials in a 50 °C water bath for 4–5 h until completely dissolved. Following the addition of 5 mL Ultima Gold, vials were agitated to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) for 5 min to eliminate noise in the form of heat and luminescence. Radioactivity was then measured for 5 min per vial. The amount of radioactivity per sample, in disintegrations per minute (dpm), was automatically calculated via Quanta-Smart software by subtracting background radiation, which is continually monitored by the scintillation counter. Frequent cleaning and monitoring of all work surfaces and equipment ensured that contamination of samples did not occur. The final dpm measures were then normalized to the weight of the sample wet mass and reported as equivalent ng BPA/g tissue or ng BPA/ml serum.

Measurement of urinary E2

Full procedures and validations for enzyme immunoassays for mouse urine were previously reported (Muir et al. 2001). Cross-reactivities for anti-E2 are: E2 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids <0.1%. Urinary E2 levels were considered with and without adjustment for urinary creatinine, which corrects for differential hydration and urinary concentration among animals, and reported as ng E2/mg creatinine and ng E2/ml urine respectively.

Statistical analyses

All statistical analyses were performed using the R software environment (R Core Team 2016). A comparison-wise error rate of $\alpha < 0.05$ was employed for all tests. Differences between treatments in Experiment 1 were analyzed by univariate analysis of variance (ANOVA) for each tissue, with Holm-Bonferroni adjustments to correct for the number of tissues (Holm 1979). Significant effects in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons. Differences between treatments in reproductive tissues and serum of animals in Experiment 2 were analyzed by t-test. Differences between urinary E2 concentrations of animals in Experiment 3 were analyzed by factorial ANOVA comparing the effects of treatment and collection time-point (repeated measures). Significant main effects or interaction in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons of treatment at each collection time-point. Data from each experiment for individual animals are provided in supplemental Tables S1–S6.

Results

Experiment 1: Measurement of ^{14}C-BPA in mice given TBBPA

This experiment was designed to determine the impact of TBBPA on the distribution of BPA. Radioactivity was measured in tissues and serum of diestrous females (Figure 1, Table S1) and males (Figure 2, Table S2) that received a sc injection of TBBPA followed by a dietary supplement of ^{14}C-BPA. Concentrations of ^{14}C-BPA in the liver and kidney are reported in Table 2. Pre-treatment with TBBPA induced a dose-dependent increase in concentrations of ^{14}C-BPA in serum and most tissues of both sexes.

Comparisons were made among the five treatments for each of nine tissues in females. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the heart, $F(4,30)=12.47$, $p<0.001$; lung, $F(4,30)=11.14$, $p<0.001$; muscle, $F(4,30)=4.23$, $p=0.024$; uterus, $F(4,30)=11.92$, $p<0.001$; ovary, $F(4,30)=16.97$, $p<0.001$; kidney, $F(4,30)=8.19$, $p<0.001$; and serum, $F(4,30)=20.41$, $p<0.001$. Multiple comparisons revealed that the vehicle-treated group differed from the 3, 9, and 27 mg groups for the heart, lung, uterus, ovaries, kidney, and serum. The vehicle-treated group also differed from the 27 mg group for the muscle.

Comparisons were made among the five treatments for each of eleven tissues in males. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the heart, $F(4,30)=8.35$, $p<0.001$; lung, $F(4,30)=7.31$, $p=0.002$; testis, $F(4,30)=11.13$, $p<0.001$; epididymis, $F(4,30)=13.76$, $p<0.001$; VC gland, $F(4,30)=4.43$, $p=0.020$; preputial gland, $F(4,30)=6.98$, $p=0.002$; liver, $F(4,30)=4.61$, $p=0.020$; kidney, $F(4,30)=8.06$, $p=0.001$; and serum, $F(4,30)=18.32$, $p<0.001$. Multiple comparisons

revealed that the vehicle-treated group differed from the 1, 3, 9, and 27 mg groups for the heart, epididymis, VC gland, kidney, and serum. The vehicle-treated group also differed from the 1 mg group for the liver; the 3, 9, and 27 mg groups for the testis; the 9 and 27 mg groups for the lung; and the 27 mg group for the preputial gland.

Figure 1 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females following sc injection of vehicle, 1, 3, 9, or 27 mg TBBPA and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=7 per dose). Difference from vehicle treatment in the same tissue: ** $p < 0.01$; + $p < 0.001$. See supplemental Table S1 for individual animal data.

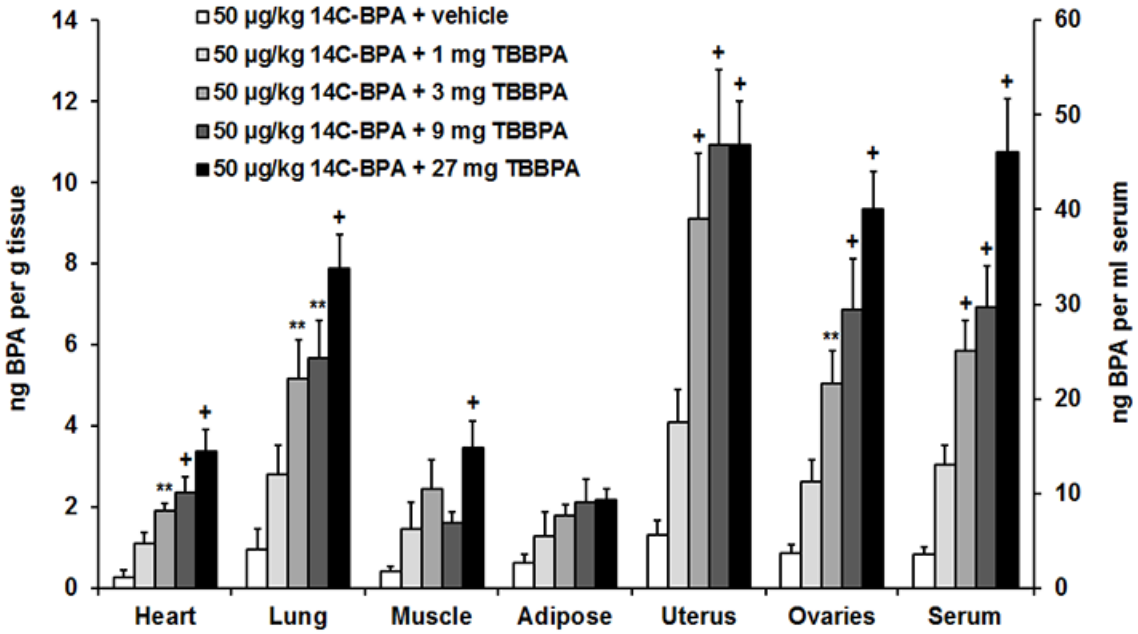

Table S1 Concentrations of ^{14}C-BPA (ng BPA/g tissue or ng BPA/ml serum) in samples from individual female mice in Experiment 1. See Figure 1 for summary data.

subject	treatment	heart	lung	muscle	adipose	uterus	ovaries	serum	liver	kidney
1	vehicle	0.233	0.756	0.256	0.379	0.714	0.531	2.619	14.492	20.002
2	vehicle	0.147	0.332	0.195	1.532	0.821	0.293	2.231	14.925	5.902
3	vehicle	1.261	3.995	0.911	1.095	3.221	1.984	7.858	46.350	41.408
4	vehicle	0.183	0.126	0.176	0.175	0.997	0.577	2.813	21.861	12.428
5	vehicle	0.000	0.511	0.811	0.822	1.851	1.105	4.754	22.258	15.145
6	vehicle	0.128	0.359	0.172	0.090	0.662	0.477	1.649	23.611	12.656
7	vehicle	0.000	0.647	0.325	0.239	1.040	1.030	3.298	23.465	15.356
8	1 mg TBBPA	2.034	4.231	5.485	0.816	3.297	2.505	15.522	13.831	48.964
9	1 mg TBBPA	0.764	0.902	0.970	0.532	2.023	1.791	9.216	27.640	18.695
10	1 mg TBBPA	0.459	1.812	0.518	0.452	3.503	0.676	7.761	14.325	12.424
11	1 mg TBBPA	1.878	5.850	1.192	1.371	8.467	4.634	20.761	36.859	50.288
12	1 mg TBBPA	0.730	1.204	0.503	0.522	2.806	1.663	8.052	8.178	23.856
13	1 mg TBBPA	0.633	1.503	0.505	0.451	3.463	2.772	11.059	18.365	61.063
14	1 mg TBBPA	1.347	4.136	0.997	4.868	5.125	4.312	18.820	44.707	54.380
15	3 mg TBBPA	2.713	6.155	6.685	1.783	14.669	6.707	33.275	33.406	109.144
16	3 mg TBBPA	1.788	6.819	1.498	2.278	6.641	4.970	24.156	43.598	86.539
17	3 mg TBBPA	1.493	1.698	1.047	0.950	3.923	1.480	15.716	17.674	26.952
18	3 mg TBBPA	2.285	8.005	2.041	1.286	13.462	7.648	39.484	40.873	74.118
19	3 mg TBBPA	1.245	1.976	1.753	1.835	12.161	3.675	20.276	17.192	41.307
20	3 mg TBBPA	2.181	6.654	2.802	3.114	7.500	4.625	21.246	41.064	101.291

21	3 mg TBBPA	1.638	4.966	1.269	1.279	5.353	6.290	21.828	59.635	53.445
22	9 mg TBBPA	2.079	3.617	1.319	5.006	9.393	4.613	22.701	25.973	43.602
23	9 mg TBBPA	3.750	7.949	1.880	1.893	18.772	10.800	36.574	12.205	69.998
24	9 mg TBBPA	2.021	8.209	1.568	1.325	10.749	6.721	33.081	17.347	56.750
25	9 mg TBBPA	3.107	6.468	2.713	2.314	12.837	10.383	42.880	12.686	78.885
26	9 mg TBBPA	0.448	1.563	0.525	0.402	3.262	1.482	8.149	5.629	14.552
27	9 mg TBBPA	2.248	7.262	1.611	1.162	7.786	6.151	29.783	17.196	33.353
28	9 mg TBBPA	2.814	4.595	1.733	2.781	13.668	8.052	34.925	11.989	40.494
29	27 mg TBBPA	4.885	10.177	2.670	1.993	14.628	11.818	54.036	12.048	92.587
30	27 mg TBBPA	4.671	9.545	2.599	2.808	8.923	8.213	73.147	15.307	93.068
31	27 mg TBBPA	3.194	9.938	2.397	2.006	11.502	11.144	46.566	12.811	44.859
32	27 mg TBBPA	2.098	4.560	6.956	1.517	8.496	6.157	28.425	20.882	82.683
33	27 mg TBBPA	4.697	7.407	3.302	2.635	14.077	11.845	47.730	10.826	69.078
34	27 mg TBBPA	2.681	8.071	4.614	3.120	11.633	9.926	40.357	13.673	50.591
35	27 mg TBBPA	1.399	5.527	1.664	1.177	7.313	6.382	32.208	31.332	108.990

Figure 2 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, testis, epididymis, VC gland, preputial gland, and serum of males following sc injection of vehicle, 1, 3, 9, or 27 mg TBBPA and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=7 per dose). Difference from vehicle treatment in the same tissue: * $p < 0.05$; ** $p < 0.01$; + $p < 0.001$. See supplemental Table S2 for individual animal data.

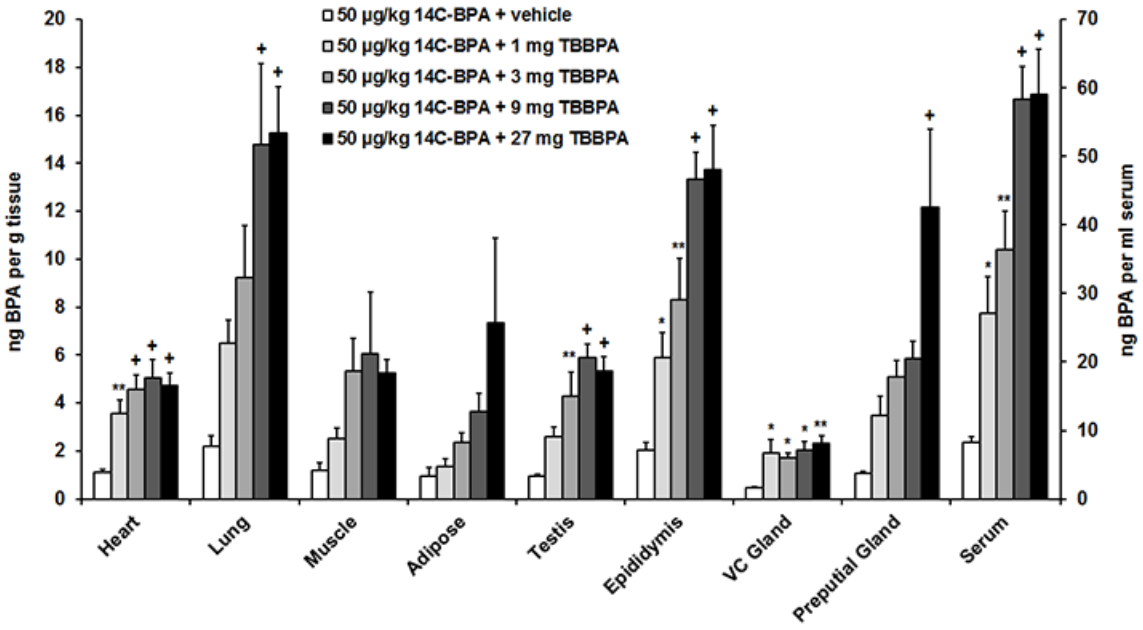

Table S2 Concentrations of ^{14}C-BPA (ng BPA/g tissue or ng BPA/ml serum) in samples from individual male mice in Experiment 1. See Figure 2 for summary data.

subject	treatment	heart	lung	muscle	adipose	testis	epididymis	vc gland	preputial gland	serum	liver	kidney
1	vehicle	1.169	0.829	1.087	1.241	1.362	3.319	0.539	1.217	12.321	1.579	104.289
2	vehicle	1.569	2.227	2.807	2.936	0.947	1.635	0.356	0.844	8.634	36.468	85.704
3	vehicle	0.834	3.146	1.036	0.059	0.934	1.831	0.402	0.778	6.306	47.520	60.633
4	vehicle	0.666	1.617	0.970	0.317	0.753	1.403	0.467	0.782	4.948	37.128	55.155
5	vehicle	0.938	2.553	0.634	1.078	0.792	2.169	0.488	1.004	8.052	71.052	91.289
6	vehicle	1.028	1.213	0.278	0.305	0.581	1.040	0.334	1.315	7.276	28.491	62.586
7	vehicle	1.493	3.907	1.546	0.649	1.197	2.955	0.596	1.406	10.089	56.068	72.679
8	1 mg TBBPA	2.325	3.969	2.096	1.424	1.584	3.362	0.954	4.221	21.634	77.870	156.518
9	1 mg TBBPA	3.979	6.309	2.314	1.181	2.895	6.618	1.614	5.145	29.589	66.537	168.217
10	1 mg TBBPA	6.112	10.701	4.757	3.057	4.804	11.653	4.653	7.196	57.722	115.109	306.214
11	1 mg TBBPA	4.657	9.239	3.295	1.001	2.105	5.653	1.356	2.458	25.417	64.794	231.410
12	1 mg TBBPA	2.553	5.174	1.157	0.689	2.506	5.147	3.328	1.780	17.074	59.108	96.774
13	1 mg TBBPA	3.421	6.136	2.529	0.911	1.624	4.655	0.734	1.908	21.634	63.770	159.988
14	1 mg TBBPA	2.023	3.911	1.505	1.235	2.569	4.187	0.850	1.546	16.201	44.343	172.363
15	3 mg TBBPA	7.381	12.555	12.308	1.732	3.496	7.496	2.444	7.114	36.865	91.564	230.441
16	3 mg TBBPA	4.713	4.779	7.599	2.007	2.541	5.550	2.001	7.505	28.037	47.050	125.754
17	3 mg TBBPA	3.524	6.835	2.885	2.655	3.744	6.306	1.960	5.092	28.328	37.688	285.433
18	3 mg TBBPA	3.517	3.331	2.572	4.350	3.366	5.715	1.146	2.668	25.126	73.148	193.642
19	3 mg TBBPA	2.412	6.002	2.495	1.287	2.373	5.060	1.372	3.106	26.484	40.360	182.337

20	3 mg TBBPA	4.395	11.305	3.316	1.808	4.399	10.074	1.858	4.280	43.462	29.104	125.799
21	3 mg TBBPA	5.945	19.762	6.114	2.663	10.167	17.992	1.217	5.735	66.842	40.124	210.936
22	9 mg TBBPA	3.812	9.131	3.908	2.175	3.978	9.557	1.217	3.519	55.103	66.452	361.260
23	9 mg TBBPA	4.851	18.681	2.994	6.323	5.007	14.255	2.055	5.444	56.558	47.082	144.859
24	9 mg TBBPA	7.867	31.588	21.440	6.666	6.576	15.864	2.364	6.286	71.401	58.241	265.302
25	9 mg TBBPA	2.956	8.358	3.182	2.998	8.632	15.398	1.413	5.384	50.544	49.095	279.520
26	9 mg TBBPA	7.176	19.503	4.468	3.039	6.566	16.988	2.169	9.234	78.968	54.223	206.125
27	9 mg TBBPA	5.689	8.084	3.439	2.836	5.137	11.235	3.901	7.172	54.812	9.637	373.746
28	9 mg TBBPA	3.134	8.069	2.824	1.549	5.303	9.901	1.202	3.838	41.230	48.975	225.949
29	27 mg TBBPA	5.811	16.816	4.356	28.070	5.369	14.042	4.134	7.580	64.125	42.254	166.300
30	27 mg TBBPA	2.102	5.145	4.918	4.474	3.612	9.962	1.668	11.778	45.305	17.530	117.205
31	27 mg TBBPA	5.770	19.305	2.575	2.679	3.687	10.918	2.023	27.806	51.999	38.747	292.201
32	27 mg TBBPA	3.918	17.371	6.504	3.649	8.060	24.125	1.969	19.657	75.282	25.281	122.526
33	27 mg TBBPA	4.133	13.946	6.327	2.314	5.303	10.309	1.679	3.410	36.574	26.231	220.041
34	27 mg TBBPA	5.040	13.711	6.003	8.433	6.266	14.702	1.979	7.696	86.050	22.313	155.858
35	27 mg TBBPA	6.222	20.516	6.146	1.730	5.094	12.035	2.652	7.163	54.424	17.900	73.806

Table 2 Mean (±SE) concentration of ^{14}C-BPA in the liver and kidney of diestrous females and males following sc injection of TBBPA and/or triclosan and subsequent dietary administration of 50 µg/kg ^{14}C-BPA. Difference from vehicle treatment in the same tissue: * $p < 0.05$; ** $p < 0.01$; + $p < 0.001$

	TBBPA Dose (mg)	Triclosan Dose (mg)	Liver (ng BPA/g)	Kidney (ng BPA/g)
Experiment 1				
Females	vehicle		23.9 ± 4.0	17.6 ± 4.3
	1		23.4 ± 5.1	38.5 ± 7.4
	3		36.2 ± 5.7	70.4 ± 11.7^{+}
	9		14.7 ± 2.4	48.2 ± 8.3*
	27		16.7 ± 2.7	77.4 ± 8.9^{+}
Males	vehicle		39.8 ± 8.3	76.0 ± 6.9
	1		70.2 ± 8.4*	184.5 ± 25.1**
	3		51.3 ± 8.5	193.5 ± 21.5**
	9		47.7 ± 6.8	265.3 ± 31.1^{+}
	27		27.2 ± 3.7	164.0 ± 27.5*
Experiment 2				
Females	vehicle		25.9 ± 2.3	15.3 ± 2.9
	0	0.33	20.8 ± 2.9	14.0 ± 1.8
	0.33	0	24.7 ± 2.4	16.7 ± 3.4
	0.33	0.33	38.2 ± 5.3	19.5 ± 1.4
Males	vehicle		39.1 ± 4.4	86.2 ± 12.0
	0	0.33	43.7 ± 2.7	114.6 ± 9.0
	0.33	0	38.6 ± 3.8	106.1 ± 24.9
	0.33	0.33	45.2 ± 4.2	119.6 ± 20.1

Experiment 2: Measurement of ^{14}C-BPA in mice given TBBPA and/or triclosan

This experiment was designed to determine whether actions of TBBPA or triclosan on the distribution of BPA would be additive when the two substances are given concurrently. Radioactivity was measured in the tissues of diestrous females (Figure 3, Table 2, Table S3) and males (Figure 4, Table 2, Table S4) that received a sc injection of TBBPA and/or triclosan followed by a dietary supplement of ^{14}C-BPA. Triclosan and TBBPA showed a greater impact on ^{14}C-BPA concentrations in serum and reproductive tissues when administered concurrently. Tests between treatment conditions in females revealed that the vehicle-treated group differed from the group given 0.33 mg TBBPA + 0.33 mg triclosan for the uterus, $t(12)=4.13$, $p=0.001$; ovaries, $t(12)=2.66$, $p=0.021$; and serum, $t(12)=4.02$, $p=0.002$. Tests between treatment conditions in males revealed that the vehicle-treated group differed from the group given 0.33 mg TBBPA + 0.33 mg triclosan for the testis, $t(10)=2.63$, $p=0.025$; epididymis, $t(10)=2.96$, $p=0.014$; and serum, $t(10)=2.89$, $p=0.016$.

Figure 3 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females following sc injection of vehicle, 0.33 mg triclosan, 0.33 mg TBBPA, or 0.33 mg triclosan + 0.33 mg TBBPA and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=7 per dose). Difference from vehicle treatment in the same tissue: * $p < 0.05$; ** $p < 0.01$. See supplemental Table S3 for individual animal data.

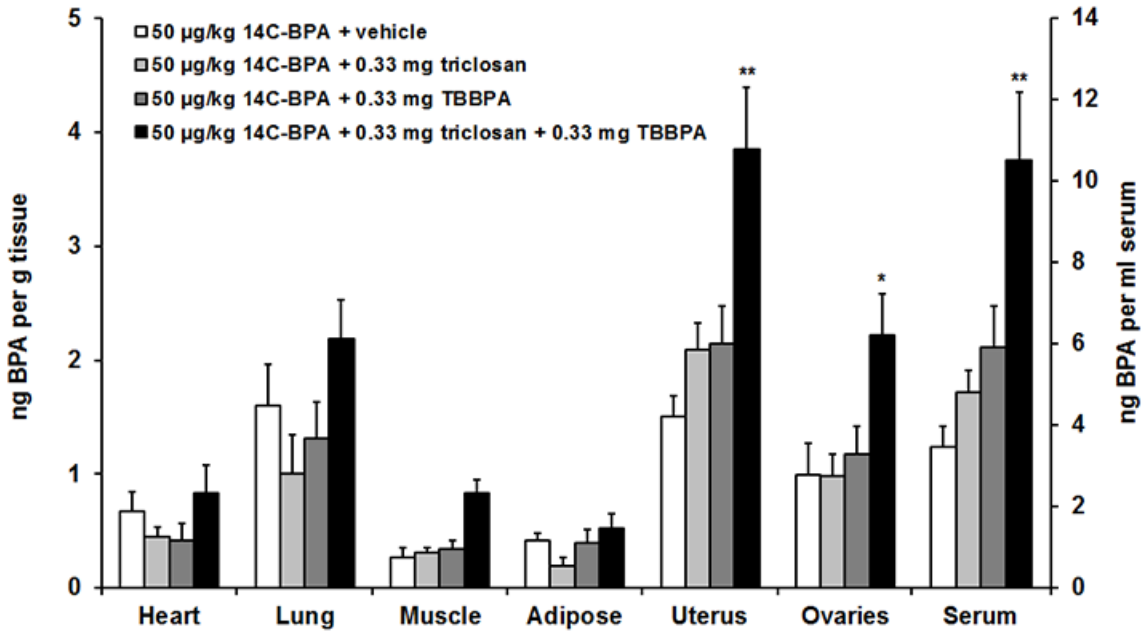

Table S3 Concentrations of ^{14}C-BPA (ng BPA/g tissue or ng BPA/ml serum) in samples from individual female mice in Experiment 2. See Figure 3 for summary data.

subject	treatment	heart	lung	muscle	adipose	uterus	ovaries	serum	liver	kidney
1	vehicle	1.465	2.592	0.524	0.542	1.532	2.080	4.321	26.792	26.353
2	vehicle	0.759	0.879	0.211	0.696	1.039	0.533	1.728	30.316	8.934
3	vehicle	0.538	3.006	0.589	0.189	1.825	0.497	2.247	23.445	6.661
4	vehicle	0.868	1.329	0.073	0.292	2.350	1.835	5.185	33.970	19.218
5	vehicle	0.601	1.461	0.265	0.413	1.474	1.165	3.975	23.524	22.231
6	vehicle	0.000	1.702	0.216	0.355	0.962	0.193	2.247	14.611	8.161
7	vehicle	0.453	0.254	0.000	0.419	1.376	0.643	4.494	28.452	15.426
8	0.33 mg triclosan	0.132	0.000	0.301	0.000	2.887	1.662	3.457	23.044	5.680
9	0.33 mg triclosan	0.516	0.000	0.504	0.198	1.891	0.941	4.148	15.987	19.251
10	0.33 mg triclosan	0.148	2.469	0.222	0.073	2.171	0.000	6.049	25.801	16.546
11	0.33 mg triclosan	0.579	1.200	0.388	0.000	1.441	1.050	3.975	17.753	14.310
12	0.33 mg triclosan	0.635	1.192	0.213	0.291	2.305	1.090	3.457	23.692	12.377
13	0.33 mg triclosan	0.617	1.571	0.198	0.596	1.252	1.252	5.531	7.854	11.453
14	0.33 mg triclosan	0.536	0.596	0.346	0.152	2.719	0.910	7.086	31.244	18.175
15	0.33 mg TBBPA	0.000	1.196	0.338	0.173	1.710	0.634	4.366	15.903	6.350
16	0.33 mg TBBPA	0.626	2.553	0.575	0.929	2.915	1.932	7.470	26.636	32.733
17	0.33 mg TBBPA	0.882	2.315	0.573	0.746	3.545	1.965	9.895	33.009	21.659
18	0.33 mg TBBPA	0.814	1.203	0.396	0.301	2.409	1.630	8.343	25.970	18.890
19	0.33 mg TBBPA	0.176	0.346	0.093	0.084	1.231	0.579	2.619	23.517	9.131
20	0.33 mg TBBPA	0.000	1.008	0.209	0.318	1.220	0.584	3.395	17.324	10.829

21	0.33 mg TBBPA	0.449	0.599	0.218	0.224	2.013	0.924	5.239	30.714	17.478
22	0.33 mg triclosan + 0.33 mg TBBPA	0.440	2.951	0.704	0.384	4.760	2.703	11.234	37.155	21.435
23	0.33 mg triclosan + 0.33 mg TBBPA	0.829	2.509	1.123	1.014	4.256	1.879	12.617	36.702	20.542
24	0.33 mg triclosan + 0.33 mg TBBPA	0.000	2.041	0.467	0.000	2.049	1.469	4.148	28.661	16.589
25	0.33 mg triclosan + 0.33 mg TBBPA	0.715	0.603	0.810	0.236	3.481	0.561	6.049	26.228	15.123
26	0.33 mg triclosan + 0.33 mg TBBPA	2.052	3.170	0.924	0.804	3.990	3.379	17.110	68.444	25.667
27	0.33 mg triclosan + 0.33 mg TBBPA	1.092	1.383	1.309	0.717	6.153	2.520	13.135	33.899	16.770
28	0.33 mg triclosan + 0.33 mg TBBPA	0.691	2.619	0.508	0.500	2.277	2.985	9.160	36.367	20.263

Figure 4 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, testis, epididymis, VC gland, preputial gland, and serum of males following sc injection of vehicle, 0.33 mg triclosan, 0.33 mg TBBPA, or 0.33 mg triclosan + 0.33 mg TBBPA and subsequent dietary administration of 50 µg/kg ^{14}C-BPA (n=6 per dose). Difference from vehicle treatment in the same tissue: * $p < 0.05$. See supplemental Table S4 for individual animal data.

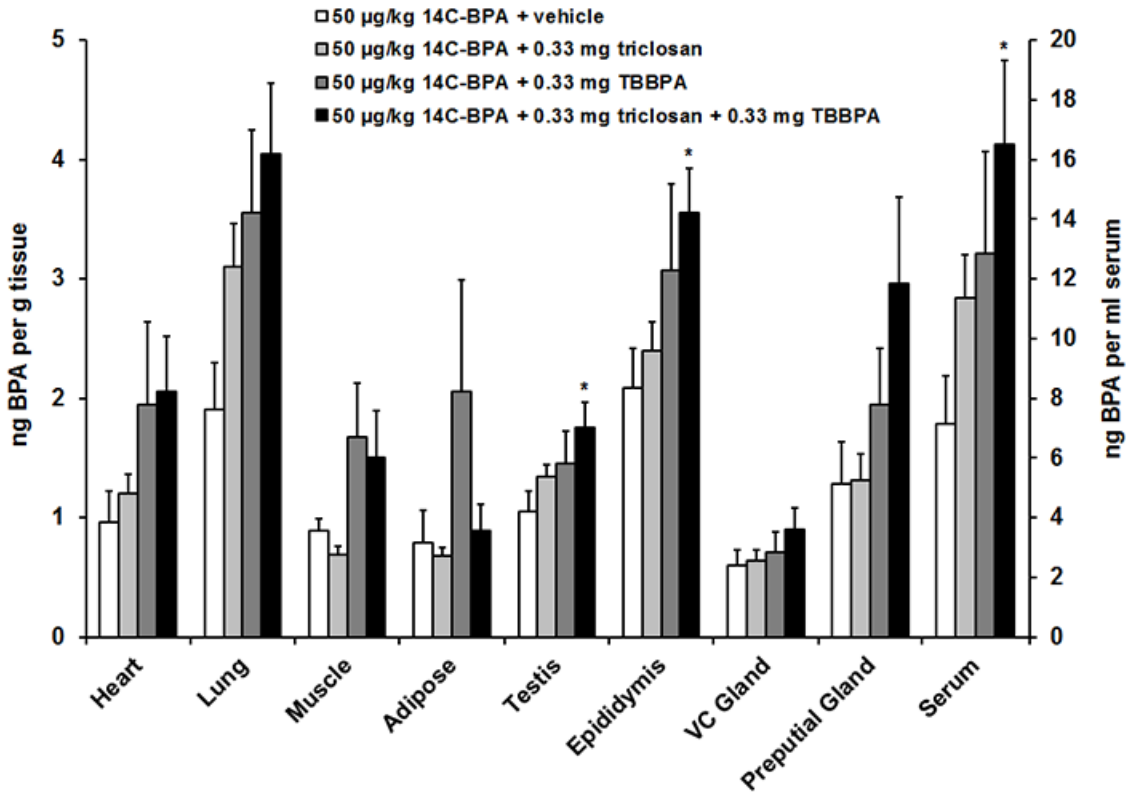

Table S4 Concentrations of ^{14}C-BPA (ng BPA/g tissue or ng BPA/ml serum) in samples from individual male mice in Experiment 2. See Figure 4 for summary data.

subject	treatment	heart	lung	muscle	adipose	testis	epididymis	vc gland	preputial gland	serum	liver	kidney
1	vehicle	0.954	1.805	0.706	0.756	1.177	2.670	0.787	0.899	5.627	33.196	103.295
2	vehicle	0.000	0.290	0.746	0.410	0.331	0.738	0.127	0.322	2.522	44.756	28.465
3	vehicle	0.729	1.460	0.920	0.630	0.898	1.723	0.421	1.070	4.172	31.384	99.608
4	vehicle	1.875	2.794	1.365	0.384	1.460	2.864	1.055	1.798	11.156	58.312	102.791
5	vehicle	1.380	2.843	0.897	2.112	1.295	2.636	0.778	2.753	12.612	31.391	101.167
6	vehicle	0.844	2.225	0.722	0.474	1.164	1.910	0.405	0.869	6.694	35.281	82.090
7	0.33 mg triclosan	1.025	1.940	0.586	0.416	0.935	1.602	0.371	0.618	7.179	39.631	83.463
8	0.33 mg triclosan	1.675	4.478	0.981	0.707	1.585	2.851	1.036	1.870	15.425	49.332	140.371
9	0.33 mg triclosan	1.004	3.197	0.642	0.554	1.172	2.835	0.656	1.252	10.962	47.466	108.249
10	0.33 mg triclosan	0.804	2.310	0.497	0.762	1.472	1.753	0.629	1.072	8.246	44.112	139.079
11	0.33 mg triclosan	0.977	3.206	0.673	0.945	1.388	2.357	0.456	1.030	10.962	32.561	113.152
12	0.33 mg triclosan	1.708	3.460	0.771	0.687	1.526	2.977	0.674	2.026	15.522	48.991	103.206
13	0.33 mg TBBPA	5.320	6.956	3.751	1.963	2.735	6.527	1.419	3.895	29.298	52.339	221.056
14	0.33 mg TBBPA	2.079	2.291	1.009	0.647	0.952	1.725	0.604	1.940	8.343	25.711	84.042
15	0.33 mg TBBPA	0.847	2.861	0.563	6.646	0.994	2.306	0.482	0.772	7.664	39.399	54.852
16	0.33 mg TBBPA	1.221	3.495	1.460	1.056	1.355	2.974	0.434	1.438	13.873	37.835	124.415
17	0.33 mg TBBPA	0.862	2.929	1.806	1.168	1.104	1.975	0.385	1.068	9.119	32.319	82.666
18	0.33 mg TBBPA	1.341	2.772	1.482	0.830	1.582	2.939	0.954	2.566	8.731	43.761	69.741
19	0.33 mg triclosan + 0.33 mg TBBPA	3.865	2.626	1.556	0.617	2.345	4.851	0.631	6.153	29.201	43.514	128.778

20	0.33 mg triclosan + 0.33 mg TBBPA	0.970	3.129	0.556	0.896	1.439	2.519	0.396	1.062	9.216	30.350	82.468
21	0.33 mg triclosan + 0.33 mg TBBPA	1.840	4.104	1.008	0.681	1.904	3.909	1.414	2.961	16.201	46.155	130.209
22	0.33 mg triclosan + 0.33 mg TBBPA	1.501	6.808	2.675	0.928	1.160	2.967	0.771	2.265	12.127	62.852	73.814
23	0.33 mg triclosan + 0.33 mg TBBPA	1.263	4.004	0.609	0.340	1.345	2.869	0.718	1.804	14.843	43.861	94.303
24	0.33 mg triclosan + 0.33 mg TBBPA	2.930	3.585	2.617	1.900	2.336	4.234	1.488	3.501	17.462	44.307	208.099

Experiment 3: Measurement of urinary E2 in mice given TBBPA

This experiment was designed to determine the impact of TBBPA on endogenous E2. Urinary E2 concentrations of diestrous females (Figure 5, Table S5) and males (Figure 6, Table S6) were measured after a sc injection of vehicle or TBBPA. Concentrations of E2 are reported for uncorrected (ng E2/ml urine) and corrected (ng E2/mg creatinine) measures. In females, ANOVA on uncorrected measures showed a significant main effect of collection time-point, $F(5,100)=17.26$, $p<0.001$, and a significant interaction, $F(5,100)=2.62$, $p=0.029$. ANOVA on creatinine-corrected measures showed significant main effects of treatment, $F(1,20)=17.12$, $p<0.001$, and collection time-point, $F(5,100)=6.98$, $p<0.001$, and a significant interaction, $F(5,100)=4.04$, $p=0.002$. Multiple comparisons revealed that the vehicle-treated females differed from the TBBPA-treated ones at 8 h after injection for the corrected measures, as well as at 10 and 12 h after injection for both the uncorrected and corrected measures. In males, ANOVA on uncorrected measures showed significant main effects of treatment, $F(1,12)=8.18$, $p=0.014$, and collection time-point, $F(5,60)=2.87$, $p=0.022$, but no significant interaction. ANOVA on corrected measures showed only a significant main effect of collection time-point, $F(5,60)=4.86$, $p<0.001$. Multiple comparisons revealed that the vehicle-treated males differed from the TBBPA-treated ones at 2 and 4 h after injection for both the uncorrected and corrected measures, as well as at 10 h after injection for the uncorrected measures.

Figure 5 Mean (+SE) concentration of urinary E2, expressed as ng E2/ml urine and ng E2/mg creatinine, following sc injection of vehicle or 1 mg TBBPA in diestrous females (n=15 per dose). Significant difference from vehicle treatment at the same time point: * $p < 0.05$; ** $p < 0.01$; + $p < 0.001$. See supplemental Table S5 for individual animal data.

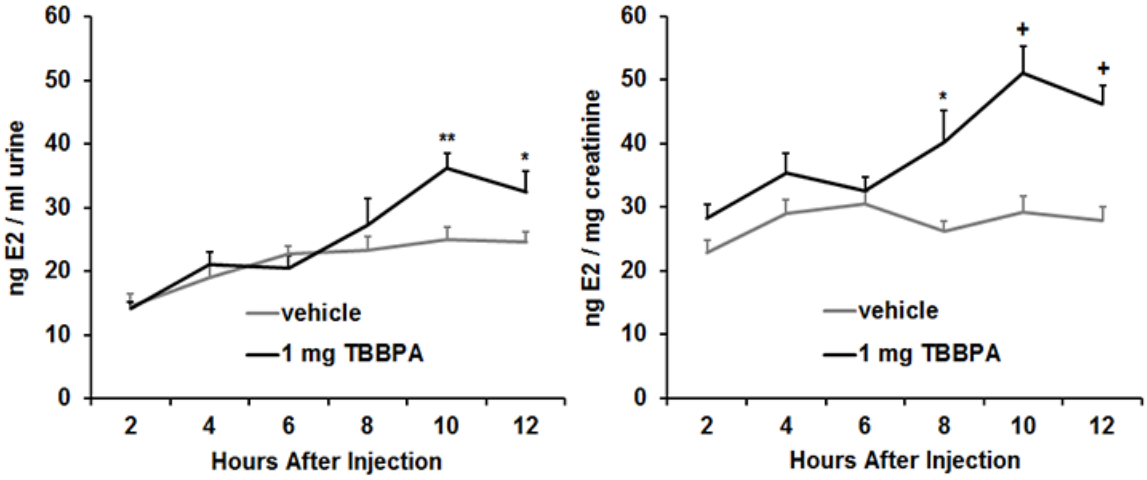

Table S5 Concentrations of E2 (ng E2/ml urine or ng E2/mg creatinine) in samples from individual female mice in Experiment 3. Blank entries are due to insufficient urine. See Figure 5 for summary data.

subject	hours after treatment	treatment	E2 (ng/ml urine)	E2 (ng/mg creatinine)
1	2	vehicle	12.543	17.967
2	2	vehicle	25.221	34.770
3	2	vehicle	11.801	19.573
4	2	vehicle	9.086	18.391
5	2	vehicle	18.558	20.884
6	2	vehicle	29.576	40.774
7	2	vehicle		
8	2	vehicle	15.080	21.186
9	2	vehicle	25.573	28.778
10	2	vehicle	7.905	14.780
11	2	vehicle	13.085	24.463
12	2	vehicle	5.376	17.707
13	2	vehicle	9.321	14.792
14	2	vehicle	8.181	25.791
15	2	vehicle	10.648	19.414
16	2	TBBPA		
17	2	TBBPA	14.016	35.142
18	2	TBBPA	17.544	35.508
19	2	TBBPA	14.685	25.508
20	2	TBBPA	19.969	33.885
21	2	TBBPA	15.769	31.060
22	2	TBBPA	12.473	26.718
23	2	TBBPA	14.815	25.140
24	2	TBBPA		
25	2	TBBPA		
26	2	TBBPA		
27	2	TBBPA	8.533	14.821
28	2	TBBPA	15.769	32.820
29	2	TBBPA	14.642	17.843

30	2	TBBPA	7.233	32.588
1	4	vehicle	11.932	18.935
2	4	vehicle	26.574	37.335
3	4	vehicle	15.350	29.447
4	4	vehicle	14.138	28.616
5	4	vehicle	23.633	25.805
6	4	vehicle	26.761	47.609
7	4	vehicle	34.698	40.280
8	4	vehicle		
9	4	vehicle	23.091	33.044
10	4	vehicle	23.621	22.198
11	4	vehicle	13.826	29.167
12	4	vehicle	8.276	23.810
13	4	vehicle	12.445	21.742
14	4	vehicle	14.472	22.520
15	4	vehicle	17.064	24.920
16	4	TBBPA	26.481	31.234
17	4	TBBPA	18.686	39.419
18	4	TBBPA	20.364	42.960
19	4	TBBPA	13.259	26.406
20	4	TBBPA	15.361	23.392
21	4	TBBPA	15.309	31.367
22	4	TBBPA	33.276	64.467
23	4	TBBPA	23.266	40.649
24	4	TBBPA		
25	4	TBBPA	26.106	43.475
26	4	TBBPA	34.417	25.064
27	4	TBBPA	19.572	41.288
28	4	TBBPA	15.464	37.009
29	4	TBBPA	24.074	31.886
30	4	TBBPA	9.624	16.815
1	6	vehicle	22.326	33.286
2	6	vehicle	28.939	49.349
3	6	vehicle	18.554	28.255
4	6	vehicle	24.074	28.683
5	6	vehicle	21.592	24.496
6	6	vehicle	21.199	33.725

7	6	vehicle	32.051	28.972
8	6	vehicle	12.251	15.644
9	6	vehicle	26.938	38.548
10	6	vehicle	26.881	30.774
11	6	vehicle	23.478	31.786
12	6	vehicle	19.941	33.027
13	6	vehicle	20.073	20.207
14	6	vehicle	20.756	31.995
15	6	vehicle	21.037	27.915
16	6	TBBPA	15.725	28.165
17	6	TBBPA	18.294	36.434
18	6	TBBPA	23.091	29.487
19	6	TBBPA	18.686	24.749
20	6	TBBPA	16.602	24.753
21	6	TBBPA	16.716	26.012
22	6	TBBPA	17.123	33.173
23	6	TBBPA	23.073	41.290
24	6	TBBPA		
25	6	TBBPA	13.298	23.798
26	6	TBBPA	43.498	45.863
27	6	TBBPA	24.316	40.275
28	6	TBBPA	19.104	42.088
29	6	TBBPA	21.615	23.924
30	6	TBBPA	15.786	35.965
1	8	vehicle	27.883	29.871
2	8	vehicle	12.767	25.593
3	8	vehicle	19.941	24.510
4	8	vehicle	28.934	36.232
5	8	vehicle	18.254	18.657
6	8	vehicle	24.402	28.424
7	8	vehicle	36.754	21.649
8	8	vehicle	10.692	19.661
9	8	vehicle	28.826	32.444
10	8	vehicle	20.411	22.592
11	8	vehicle	42.238	37.941
12	8	vehicle	16.436	20.203
13	8	vehicle	16.283	23.992

14	8	vehicle	26.114	29.391
15	8	vehicle	18.733	21.821
16	8	TBBPA	20.275	27.450
17	8	TBBPA	27.679	48.238
18	8	TBBPA	32.791	34.036
19	8	TBBPA	18.078	28.526
20	8	TBBPA	15.497	22.833
21	8	TBBPA	13.900	22.465
22	8	TBBPA	7.243	13.695
23	8	TBBPA	20.756	23.762
24	8	TBBPA	77.704	84.324
25	8	TBBPA	21.863	48.263
26	8	TBBPA	37.558	49.729
27	8	TBBPA	38.362	52.911
28	8	TBBPA	27.222	56.333
29	8	TBBPA	22.918	30.344
30	8	TBBPA	26.004	61.508
1	10	vehicle	22.423	18.553
2	10	vehicle	22.342	33.618
3	10	vehicle	21.170	25.026
4	10	vehicle	30.123	33.235
5	10	vehicle	24.672	19.915
6	10	vehicle	32.502	40.597
7	10	vehicle		
8	10	vehicle	13.293	25.889
9	10	vehicle	22.423	20.332
10	10	vehicle	22.101	25.225
11	10	vehicle	44.462	53.517
12	10	vehicle	26.103	32.001
13	10	vehicle	16.532	23.794
14	10	vehicle	22.423	20.615
15	10	vehicle	29.304	35.272
16	10	TBBPA	45.261	50.782
17	10	TBBPA	35.421	63.389
18	10	TBBPA	33.990	40.912
19	10	TBBPA	34.553	38.122
20	10	TBBPA	32.502	41.378

21	10	TBBPA	21.942	30.908
22	10	TBBPA	40.558	92.621
23	10	TBBPA	31.346	41.504
24	10	TBBPA	61.584	59.079
25	10	TBBPA	41.991	47.114
26	10	TBBPA	29.886	50.740
27	10	TBBPA	36.622	69.286
28	10	TBBPA	35.568	52.330
29	10	TBBPA	32.370	29.760
30	10	TBBPA	28.825	57.220
1	12	vehicle	21.735	18.126
2	12	vehicle	27.419	41.866
3	12	vehicle	20.326	18.852
4	12	vehicle	27.419	28.198
5	12	vehicle	20.540	19.051
6	12	vehicle	26.003	25.935
7	12	vehicle		
8	12	vehicle	21.504	22.825
9	12	vehicle	29.621	25.673
10	12	vehicle	20.904	30.510
11	12	vehicle	26.497	37.838
12	12	vehicle	29.971	31.309
13	12	vehicle	17.031	26.005
14	12	vehicle	39.067	41.467
15	12	vehicle	16.002	23.355
16	12	TBBPA	29.621	52.498
17	12	TBBPA	25.905	44.715
18	12	TBBPA		
19	12	TBBPA		
20	12	TBBPA	43.324	54.773
21	12	TBBPA	42.574	55.964
22	12	TBBPA	55.096	60.419
23	12	TBBPA	24.498	30.391
24	12	TBBPA		
25	12	TBBPA	33.374	43.016
26	12	TBBPA		
27	12	TBBPA	35.778	47.031

28	12	TBBPA	26.298	42.097
29	12	TBBPA	20.326	32.538
30	12	TBBPA	20.904	45.602

Figure 6 Mean (+SE) concentration of urinary E2, expressed as ng E2/ml urine and ng E2/mg creatinine, following sc injection of vehicle or 1 mg TBBPA in males (n=15 per dose). Significant difference from vehicle treatment at the same time point: * $p < 0.05$; ** $p < 0.01$; + $p < 0.001$. See supplemental Table S6 for individual animal data.

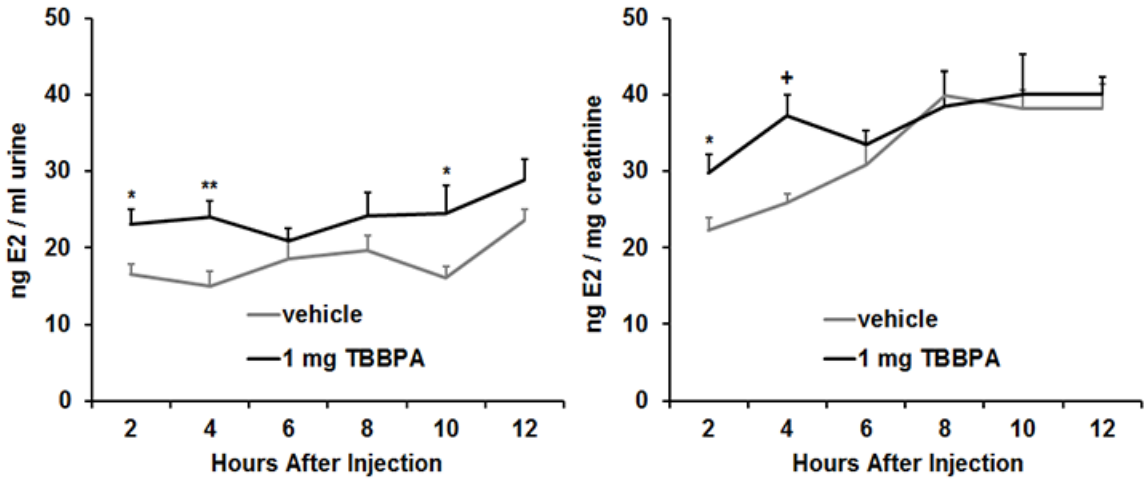

Table S6 Concentrations of E2 (ng E2/ml urine or ng E2/mg creatinine) in samples from individual male mice in Experiment 3. Blank entries are due to insufficient urine. See Figure 6 for summary data.

subject	hours after treatment	treatment	E2 (ng/ml urine)	E2 (ng/mg creatinine)
1	2	vehicle		
2	2	vehicle	13.914	19.683
3	2	vehicle	14.921	21.531
4	2	vehicle	17.969	22.342
5	2	vehicle		
6	2	vehicle	15.341	16.505
7	2	vehicle	6.118	12.629
8	2	vehicle	16.277	22.153
9	2	vehicle	12.993	18.749
10	2	vehicle	15.677	28.300
11	2	vehicle	23.027	25.539
12	2	vehicle	18.931	22.015
13	2	vehicle		
14	2	vehicle	20.229	33.184
15	2	vehicle	23.350	25.897
16	2	TBBPA	22.631	37.126
17	2	TBBPA	24.354	28.321
18	2	TBBPA	23.761	45.160
19	2	TBBPA	26.630	30.476
20	2	TBBPA	25.054	28.672
21	2	TBBPA		
22	2	TBBPA	32.070	33.023
23	2	TBBPA	14.876	21.465
24	2	TBBPA	25.323	28.086
25	2	TBBPA		
26	2	TBBPA	12.149	20.883
27	2	TBBPA		
28	2	TBBPA	16.536	22.505
29	2	TBBPA	35.191	42.292

30	2	TBBPA	19.056	20.201
1	4	vehicle		
2	4	vehicle		
3	4	vehicle	15.532	29.520
4	4	vehicle	14.428	20.016
5	4	vehicle	20.571	24.722
6	4	vehicle	16.328	23.098
7	4	vehicle	8.500	20.486
8	4	vehicle		
9	4	vehicle	11.838	25.925
10	4	vehicle	13.305	28.276
11	4	vehicle	25.780	29.041
12	4	vehicle	12.084	28.109
13	4	vehicle	7.822	24.535
14	4	vehicle	6.809	23.398
15	4	vehicle	27.555	34.238
16	4	TBBPA	26.060	45.890
17	4	TBBPA	14.516	26.879
18	4	TBBPA		
19	4	TBBPA	22.476	31.180
20	4	TBBPA	16.074	28.306
21	4	TBBPA		
22	4	TBBPA		
23	4	TBBPA	33.162	48.779
24	4	TBBPA	26.669	44.708
25	4	TBBPA	17.311	29.712
26	4	TBBPA	25.004	39.181
27	4	TBBPA		
28	4	TBBPA	17.563	26.373
29	4	TBBPA	37.842	46.223
30	4	TBBPA	27.107	43.421
1	6	vehicle		
2	6	vehicle	13.523	24.997
3	6	vehicle	22.748	35.646
4	6	vehicle	16.702	29.366
5	6	vehicle	21.306	40.422
6	6	vehicle		

7	6	vehicle	11.966	17.969
8	6	vehicle	39.950	39.982
9	6	vehicle	12.323	16.757
10	6	vehicle	15.618	36.330
11	6	vehicle	31.904	36.493
12	6	vehicle	10.337	24.848
13	6	vehicle	9.807	20.798
14	6	vehicle	8.691	28.503
15	6	vehicle	25.612	48.592
16	6	TBBPA	14.373	24.670
17	6	TBBPA	9.227	22.181
18	6	TBBPA	26.778	37.843
19	6	TBBPA	25.924	39.757
20	6	TBBPA	14.572	25.010
21	6	TBBPA		
22	6	TBBPA	19.756	30.957
23	6	TBBPA	28.244	45.241
24	6	TBBPA	23.378	34.388
25	6	TBBPA	22.837	32.920
26	6	TBBPA	14.774	31.330
27	6	TBBPA	18.895	35.847
28	6	TBBPA	30.706	35.690
29	6	TBBPA	21.614	34.799
30	6	TBBPA	20.810	38.919
1	8	vehicle	14.315	35.338
2	8	vehicle	26.305	35.724
3	8	vehicle	24.671	42.690
4	8	vehicle	13.889	43.584
5	8	vehicle	32.975	57.059
6	8	vehicle	11.149	15.758
7	8	vehicle	24.769	41.818
8	8	vehicle	13.796	32.889
9	8	vehicle	20.270	50.039
10	8	vehicle	26.412	26.529
11	8	vehicle	14.658	53.211
12	8	vehicle	6.386	48.587
13	8	vehicle		

14	8	vehicle	21.047	46.950
15	8	vehicle	24.868	29.203
16	8	TBBPA		
17	8	TBBPA	47.769	68.918
18	8	TBBPA		
19	8	TBBPA	16.427	16.500
20	8	TBBPA	20.119	42.170
21	8	TBBPA		
22	8	TBBPA	17.017	36.778
23	8	TBBPA	26.954	49.087
24	8	TBBPA	22.546	28.398
25	8	TBBPA	13.172	30.357
26	8	TBBPA	24.188	30.465
27	8	TBBPA		
28	8	TBBPA	25.573	38.495
29	8	TBBPA	28.198	44.370
30	8	TBBPA		
1	10	vehicle		
2	10	vehicle		
3	10	vehicle	14.123	39.028
4	10	vehicle	19.243	26.655
5	10	vehicle	18.217	38.183
6	10	vehicle	21.780	41.860
7	10	vehicle	17.762	40.938
8	10	vehicle		
9	10	vehicle	16.149	35.262
10	10	vehicle	22.566	50.850
11	10	vehicle		
12	10	vehicle	11.742	40.822
13	10	vehicle	10.231	25.502
14	10	vehicle	6.584	32.519
15	10	vehicle	18.008	48.305
16	10	TBBPA		
17	10	TBBPA	7.309	13.310
18	10	TBBPA	17.509	60.406
19	10	TBBPA	42.615	64.148
20	10	TBBPA	13.259	23.529

21	10	TBBPA		
22	10	TBBPA	19.675	30.958
23	10	TBBPA		
24	10	TBBPA		
25	10	TBBPA	18.425	31.457
26	10	TBBPA	22.803	35.492
27	10	TBBPA		
28	10	TBBPA	34.841	50.856
29	10	TBBPA	29.729	36.575
30	10	TBBPA	39.323	54.040
1	12	vehicle	20.567	21.866
2	12	vehicle	22.332	34.758
3	12	vehicle	27.486	45.816
4	12	vehicle	27.486	39.306
5	12	vehicle		
6	12	vehicle	31.118	39.669
7	12	vehicle	20.637	34.400
8	12	vehicle	27.896	27.195
9	12	vehicle	22.024	37.602
10	12	vehicle	31.844	63.619
11	12	vehicle	22.409	34.879
12	12	vehicle	12.736	24.742
13	12	vehicle		
14	12	vehicle	15.312	48.454
15	12	vehicle	23.786	44.969
16	12	TBBPA	30.413	45.333
17	12	TBBPA	17.377	24.356
18	12	TBBPA	24.559	44.066
19	12	TBBPA	34.025	35.637
20	12	TBBPA	26.206	32.814
21	12	TBBPA		
22	12	TBBPA	40.308	44.886
23	12	TBBPA	23.786	47.520
24	12	TBBPA	51.933	52.070
25	12	TBBPA	18.607	32.557
26	12	TBBPA	25.549	48.303
27	12	TBBPA		

28	12	TBBPA	31.844	47.466
29	12	TBBPA	33.364	32.983
30	12	TBBPA	17.598	32.084

Discussion

These data show that TBBPA greatly magnifies concentrations of BPA in serum and tissues, and that it elevates urinary concentrations of E2. When animals received an oral dose of ^{14}C-BPA, radioactivity was greater in serum, reproductive tissues, and elsewhere in female and male mice that were pre-treated with 1–27 mg TBBPA. Radioactivity was also greater in serum and reproductive tissues of mice given 0.33 mg TBBPA + 0.33 mg triclosan. Urinary E2 concentrations were elevated in animals given 1 mg TBBPA. Our novel findings that TBBPA modulates E2 and BPA concentrations underscore a concern (Osimitz et al. 2014) that molecular modeling and *in vitro* studies may not address biological activity *in vivo*.

There are several potential mechanisms through which TBBPA, triclosan, BPA, and E2 could interact. These include direct actions at ER, transport proteins in blood, and enzymes involved in steroid synthesis and metabolism. There is conflicting evidence regarding direct binding to ER of TBBPA (Lee et al. 2012; Li et al. 2010; Molina-Molina et al. 2013; Suzuki et al. 2013) and triclosan (Gee et al. 2008; Henry and Fair 2013; Stoker et al. 2010). Insofar as there is such binding, actions of TBBPA or triclosan would be competitive with binding of ^{14}C-BPA, producing an opposite effect to that observed in Experiments 1 and 2. Similarly, competition for transport proteins in blood would presumably reduce ^{14}C-BPA concentrations in serum and tissues. Our findings in Experiments 1 and 2 are much more consistent with competition among TBBPA, triclosan, and ^{14}C-BPA for metabolic enzymes. Enzymes of particular interest are those involved in phase II metabolism, including UGT and SULT (Dumas and Diorio 2011;

Wikoff et al. 2016). The major metabolite of BPA in rodents is the monoglucuronide conjugate resulting from interaction with hepatic UGT 2B1 and potentially other isoforms (Inoue et al. 2001; Kurebayashi et al. 2010; Yokota et al. 1999; Zalko et al. 2003). Other metabolites of BPA in rodents include the monosulfate conjugate resulting from interaction with SULT 1A1 (Yalcin et al. 2016; Zalko et al. 2003), as well as the diglucuronide (Zalko et al. 2003), disulfate (Yalcin et al. 2016), and glucuronide/sulfate (Inoue et al. 2016) diconjugates. Sulfate and glucuronide conjugates of TBBPA (Borghoff et al. 2016) and triclosan (Fang et al. 2014) are also observed in rodents. TBBPA can inhibit the activity of SULT 1E1 and SULT 1A1 (Gosavi et al. 2013; Hamers et al. 2006; Harju et al. 2007; Kester et al. 2002) and reduce expression of genes in the liver that encode SULT 1E1 and SULT 2A1 (Sanders et al. 2016). Triclosan can inhibit sulfonation and glucuronidation of BPA in human liver fractions (Wang et al. 2004). Our findings in Experiment 3 can be explained by competition between TBBPA and E2 for metabolic enzymes. In addition to phase II metabolism described above, phase I metabolism involving CYP and 17β-HSD is important for estrogen metabolism (Dumas and Diorio 2011; Wikoff et al. 2016). TBBPA can inhibit CYP 2C9 and CYP 3A4 activities (Ames 2013) and 17β-HSD4 activity (NIH 2010) in human liver fractions. TBBPA can reduce expression of genes in the liver that encode 17β-HSD, but increase expression of genes that encode certain CYP isoforms (Sanders et al. 2016). Our findings in Experiment 3 can also be explained by actions of TBBPA on enzymes involved in steroid synthesis. One study found TBBPA upregulates aromatase expression and activity in human choriocarcinoma cells for up to 72 h *in vitro* (Honkisz and Wójtowicz 2015).

The latency of TBBPA actions on E2, up to 8–12 h in females, could be attributed to increased E2 biosynthesis.

We found that TBBPA magnified concentrations of ^{14}C-BPA in the heart, lungs, kidneys, and blood serum of mice, as well as the uterus and ovaries of females and the testes, epididymides, VC glands, and preputial glands of males. The impact of 1–27 mg TBBPA on ^{14}C-BPA concentrations in tissues and serum is consistent with that previously shown for 0.6–18 mg triclosan (Pollock et al. 2014), except that TBBPA appears to have larger effects. This is especially evident in males, as pre-treatment with triclosan elevated ^{14}C-BPA concentrations in only the epididymides and blood serum (Pollock et al. 2014). The greatest impact of TBBPA on ^{14}C-BPA concentrations was in the lungs, reproductive tissues, kidneys, and blood serum. The localization of ^{14}C-BPA to the lungs and reproductive tissues is consistent with the high expression of ERα and ERβ in these tissues (Couse et al. 1997; Kuiper et al. 1997). Of the tissue samples collected, the highest concentrations of ^{14}C-BPA were in the liver and kidney. This is consistent with previous studies of the distribution of BPA at doses ranging from 0.5 to 100,000 µg/kg (Kim et al. 2004; Kurebayashi et al. 2005; Pollock and deCatanzaro 2014). These organs are involved in the metabolism and excretion of ingested BPA, and radioactivity in these tissues does not necessarily reflect tissue deposition of ^{14}C-BPA. Concentrations of ^{14}C-BPA were greater in males than females in most non-reproductive tissues and blood serum. In vehicle-treated animals in Experiment 1, average ^{14}C-BPA concentrations of males were greater than those of females for the heart (394%), lungs (230%), muscle (294%), adipose (152%), serum (228%), liver (167%), and kidney

(433%). This is consistent with the distribution of 100 µg/kg BPA in certain tissues of male and female rats (Kurebayashi et al. 2005), and may be explained by differences in BPA metabolism, as evidenced by sex- and tissue-specific expression of numerous UGT isoforms (Buckley and Klaassen 2007).

Greater concentrations of urinary E2 following TBBPA administration were most evident in females around 8–12 h post-injection, but were also observed in males around 2–4 h post-injection. This discrepancy in latency between males and females may be influenced by differences in the metabolism of TBBPA and/or estrogen synthesis. The rate of TBBPA glucuronide production is faster in male rat liver fractions (Zalko et al. 2006), whereas aromatase expression is greater in the ovaries than in the testes (Golovine et al. 2003). Taken together, this may hasten the influence of TBBPA on E2 concentrations in males but result in greater effects of TBBPA on E2 concentrations in females. Slight but persistent elevations in E2 can lead to adverse reproductive and health outcomes in mammals. In mice, heightened E2 levels can prevent intrauterine blastocyst implantation and cause pregnancy failure (Thorpe et al. 2013). In humans, elevated E2 from hormone-replacement therapy correlates with increased risk of breast, endometrial, and ovarian cancer (Million Women Study Collaborators 2003, 2005, 2007).

Data from the 2011–2012 U.S. National Health and Nutrition Examination Survey (NHANES) indicated that 72% of human urine samples contain detectable triclosan concentrations ranging from 2.3 to 3,830 µg/L (Han et al. 2016). Although NHANES does not report TBBPA concentrations in urine, TBBPA was detected in 93% of plasma samples and 89% of urine samples in a study of 140 healthy adults in China (Ho et al.

2017). Some published reports have estimated daily TBBPA exposure levels in the range of 3.2E-7 to 1.95E-4 mg/kg/day (Health Canada 2013; NTP 2014; Wikoff et al. 2015). However, these exposure estimates are derived from concentrations of TBBPA in environmental media and may not account for all exposure pathways. Furthermore, interactions among chemicals may influence their distribution, metabolism, and excretion, as evidenced in our data. One study suggested that disruption of homeostatic control of TBBPA and estrogen conjugation is unlikely in humans since the doses required to produce uterine tumors in rodents are orders of magnitude greater than exposure estimates in humans (Borghoff et al. 2016). However, our data show clear *in vivo* interaction between TBBPA and triclosan, indicating that it is not appropriate to consider only one chemical in isolation. Inhibition of enzymes involved in estrogen metabolism has been shown for a number of environmental chemicals and their metabolites, including parabens (Ozaki et al. 2016; Prusakiewicz et al. 2007), phthalates (Ozaki et al. 2016), polychlorinated biphenyls (Kester et al. 2000; Wang and James 2007), and polyhalogenated aromatic hydrocarbons (Kester et al. 2002). Given the potential adverse reproductive and carcinogenic outcomes of persistently elevated estrogenic activity, these findings demonstrate the importance of considering multiple toxicants when determining regulatory exposure limits.

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References

Abdallah MA-E. 2016. Environmental occurrence, analysis and human exposure to the flame retardant tetrabromobisphenol-A (TBBP-A)-A review. Environ Int 94:235–250, doi:10.1016/j.envint.2016.05.026.

Abdallah MA-E, Harrad S. 2011. Tetrabromobisphenol-A, hexabromocyclododecane and its degradation products in UK human milk: Relationship to external exposure. Environ Int 37:443–448, doi:10.1016/j.envint.2010.11.008.

Ames B. 2013. Tetrabrominated bisphenol A inhibition studies on six cytochrome P450 enzymes in human liver microsomes. California State University (dissertation).

Beck KR, Sommer TJ, Schuster D, Odermatt A. 2016. Evaluation of tetrabromobisphenol A effects on human glucocorticoid and androgen receptors: A comparison of results from human- with yeast-based *in vitro* assays. Toxicology 370:70–77, doi:10.1016/j.tox.2016.09.014.

Berger RG, Foster WG, deCatanzaro D. 2010. Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. Reprod Toxicol 30:393–400, doi:10.1016/j.reprotox.2010.06.006.

Berger RG, Hancock T, deCatanzaro D. 2007. Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. Reprod Toxicol 23:138–144, doi:10.1016/j.reprotox.2006.09.005.

Berger RG, Shaw J, deCatanzaro D. 2008. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and

17β-estradiol. Reprod Toxicol 26:94–99, doi:10.1016/j.reprotox.2008.06.007.

Borghoff SJ, Wikoff D, Harvey S, Haws L. 2016. Dose- and time-dependent changes in tissue levels of tetrabromobisphenol A (TBBPA) and its sulfate and glucuronide conjugates following repeated administration to female Wistar Han Rats. Toxicol Reports 3:190–201, doi:10.1016/j.toxrep.2016.01.007.

Borman ED, Foster WG, Greenacre MKE, Muir CC, deCatanzaro D. 2015. Stress lowers the threshold dose at which bisphenol A disrupts blastocyst implantation, in conjunction with decreased uterine closure and e-cadherin. Chem Biol Interact 237:87–95, doi:10.1016/j.cbi.2015.05.012.

Buckley DB, Klaassen CD. 2007. Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. Drug Metab Dispos 35:121–127, doi:10.1124/dmd.106.012070.

Byers SL, Wiles M V., Dunn SL, Taft RA. 2012. Mouse estrous cycle identification tool and images. PLoS One 7:e35538, doi:10.1371/journal.pone.0035538.

Cariou R, Antignac J-P, Zalko D, Berrebi A, Cravedi J-P, Maume D, et al. 2008. Exposure assessment of French women and their newborns to tetrabromobisphenol-A: Occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. Chemosphere 73:1036–1041, doi:10.1016/j.chemosphere.2008.07.084.

Colnot T, Kacew S, Dekant W. 2014. Mammalian toxicology and human exposures to the flame retardant 2,2′,6,6′-tetrabromo-4,4′-isopropylidenediphenol (TBBPA): Implications for risk assessment. Arch Toxicol 88:553–573, doi:10.1007/s00204-013-1180-8.

Couse JF, Lindzey J, Grandien K, Gustafsson J, Korach KS. 1997. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 138:4613–4621, doi:10.1210/en.138.11.4613.

Crawford BR, deCatanzaro D. 2012. Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A. Reprod Toxicol 34:607–613, doi:10.1016/j.reprotox.2012.09.008.

deCatanzaro D, Muir C, Beaton EA, Jetha M. 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69:687–696, doi:10.1016/j.steroids.2004.07.002.

deCatanzaro D, Muir C, Beaton E, Jetha M, Nadella K. 2003. Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 126:407–414, doi:10.1530/reprod/126.3.407.

deCatanzaro D, Pollock T. 2016. Absorption and distribution of estradiol from male seminal emissions during mating. J Endocrinol 231:245–257, doi:10.1530/JOE-16-0247.

Dorosh A, Děd L, Elzeinová F, Pěknicová J. 2011. Assessing oestrogenic effects of brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on MCF-7 cells. Folia Biol 57:35–39.

Dumas I, Diorio C. 2011. Estrogen pathway polymorphisms and mammographic density. Anticancer Res 31:4369–4386.

EFSA (European Food Safety Authority). 2015. Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA J 13:3978, doi:10.2903/j.efsa.2015.3978.

Fang J-L, Vanlandingham M, da Costa GG, Beland FA. 2014. Absorption and metabolism of triclosan after application to the skin of B6C3F1 mice. Environ Toxicol 31:609–623.

Fujii Y, Nishimura E, Kato Y, Harada KH, Koizumi A, Haraguchi K. 2014. Dietary exposure to phenolic and methoxylated organohalogen contaminants in relation to their concentrations in breast milk and serum in Japan. Environ Int 63:19–25, doi:10.1016/j.envint.2013.10.016.

Gee RH, Charles A, Taylor N, Darbre PD. 2008. Oestrogenic and androgenic activity of triclosan in breast cancer cells. J Appl Toxicol 28:78–91, doi:10.1002/jat.1316.

Golovine K, Schwerin M, Vanselow J. 2003. Three different promoters control expression of the aromatase cytochrome P450 gene (*Cyp19*) in mouse gonads and brain. Biol Reprod 68:978–984, doi:10.1095/biolreprod.102.008037.

Gosavi RA, Knudsen GA, Birnbaum LS, Pedersen LC. 2013. Mimicking of estradiol binding by flame retardants and their metabolites: A crystallographic analysis. Environ Health Perspect 121:1194–1199, doi:10.1289/ehp.1306902.

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MHA, Andersson PL, et al. 2006. *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol Sci 92:157–173, doi:10.1093/toxsci/kfj187.

Han C, Lim YH, Hong YC. 2016. Ten-year trends in urinary concentrations of triclosan

and benzophenone-3 in the general U.S. population from 2003 to 2012. Environ Pollut 208:803–810, doi:10.1016/j.envpol.2015.11.002.

Harju M, Hamers T, Kamstra JH, Sonneveld E, Boon JP, Tysklind M, et al. 2007. Quantitative structure–activity relationship modeling on *in vitro* endocrine effects and metabolic stability involving 26 selected brominated flame retardants. Environ Toxicol Chem 26:816–826, doi:10.1897/06-308R.1.

Health Canada. 2013. Screening assessment report: Tetrabromobisphenol A (TBBPA; CAS RN 79-94-7), TBBPA bis(2-hydroxyethyl ether) (CAS RN 4162-45-2), and TBBPA bis(allyl ether) (CAS RN 25327-89-3). ISBN 978-1-100-22898-3. Available: http://www.ec.gc.ca/ese-ees/BEE093E4-8387-4790-A9CD-C753B3E5BFAD/FSAR_TBBPA_EN.pdf [accessed 10 December 2016].

Henry ND, Fair PA. 2013. Comparison of *in vitro* cytotoxicity, estrogenicity and anti-estrogenicity of triclosan, perfluorooctane sulfonate and perfluorooctanoic acid. J Appl Toxicol 33:265–272, doi:10.1002/jat.1736.

Ho KL, Yuen KK, Yau MS, Murphy MB, Wan Y, Fong BMW, et al. 2017. Glucuronide and sulfate conjugates of tetrabromobisphenol A (TBBPA): Chemical synthesis and correlation between their urinary levels and plasma TBBPA content in voluntary human donors. Environ Int 98:46–53, doi:10.1016/j.envint.2016.09.018.

Holm S. 1979. A simple sequentially rejective multiple test procedure. Scand J Stat 6:65–70, doi:10.2307/4615733.

Honkisz E, Wójtowicz AK. 2015. Modulation of estradiol synthesis and aromatase activity in human choriocarcinoma JEG-3 cells exposed to tetrabromobisphenol A.

Toxicol In Vitro 29:44–50, doi:10.1016/j.tiv.2014.09.003.

Huang GY, Ying GG, Liang YQ, Zhao JL, Yang B, Liu S, et al. 2013. Hormonal effects of tetrabromobisphenol A using a combination of *in vitro* and *in vivo* assays. Comp Biochem Physiol C Toxicol Pharmacol 157:344–351, doi:10.1016/j.cbpc.2013.03.003.

Inoue H, Kemanai S, Sano C, Kato S, Yokota H, Iwano H. 2016. Bisphenol A glucuronide/sulfate diconjugate in perfused liver of rats. J Vet Med Sci 78:733–737, doi:10.1292/jvms.15-0573.

Inoue H, Yokota H, Makino T, Yuasa A, Kato S. 2001. Bisphenol A glucuronide, a major metabolite in rat bile after liver perfusion. Drug Metab Dispos 29:1084–1087.

James MO, Ambadapadi S, Falany C. 2015. Triclosan inhibits the activity of expressed human sulfotransferases (SULTs) towards their diagnostic substrates. FASEB J 29:622 (Abstract).

James MO, Li W, Summerlot DP, Rowland-Faux L, Wood CE. 2010. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ Int 36:942–949, doi:10.1016/j.envint.2009.02.004.

Johnson-Restrepo B, Adams DH, Kannan K. 2008. Tetrabromobisphenol A (TBBPA) and hexabromocyclododecanes (HBCDs) in tissues of humans, dolphins, and sharks from the United States. Chemosphere 70:1935–1944, doi:10.1016/j.chemosphere.2007.10.002.

Kester MHA, Bulduk S, Tibboel D, Meinl W, Glatt H, Falany CN, et al. 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: A novel

pathway explaining the estrogenic activity of PCBs. Endocrinology 141:1897–1900, doi:10.1210/endo.141.5.7530.

Kester MHA, Bulduk S, van Toor H, Tibboel D, Meinl W, Glatt H, et al. 2002. Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J Clin Endocrinol Metab 87:1142–1150, doi:10.1210/jcem.87.3.8311.

Kim CS, Sapienza PP, Ross IA, Johnson W, Luu HMD, Hutter JC. 2004. Distribution of bisphenol A in the neuroendocrine organs of female rats. Toxicol Ind Health 20:41–50, doi:10.1191/0748233704th186oa.

Kim UJ, Lee IS, Oh JE. 2016. Occurrence, removal and release characteristics of dissolved brominated flame retardants and their potential metabolites in various kinds of wastewater. Environ Pollut 218:551–557, doi:10.1016/j.envpol.2016.07.037.

Knudsen GA, Hughes MF, McIntosh KL, Sanders JM, Birnbaum LS. 2015. Estimation of tetrabromobisphenol A (TBBPA) percutaneous uptake in humans using the parallelogram method. Toxicol Appl Pharmacol 289:323–329, doi:10.1016/j.taap.2015.09.012.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138: 863–870.

Kurebayashi H, Nagatsuka SI, Nemoto H, Noguchi H, Ohno Y. 2005. Disposition of low

doses of ^{14}C-bisphenol A in male, female, pregnant, fetal, and neonatal rats. Arch Toxicol 79:243–252, doi:10.1007/s00204-004-0628-2.

Kurebayashi H, Okudaira K, Ohno Y. 2010. Species difference of metabolic clearance of bisphenol A using cryopreserved hepatocytes from rats, monkeys and humans. Toxicol Lett 198:210–215, doi:10.1016/j.toxlet.2010.06.017.

La Guardia MJ, Hale RC. 2015. Halogenated flame-retardant concentrations in settled dust, respirable and inhalable particulates and polyurethane foam at gymnastic training facilities and residences. Environ Int 79:106–114, doi:10.1016/j.envint.2015.02.014.

Lai DY, Kacew S, Dekant W. 2015. Tetrabromobisphenol A (TBBPA): Possible modes of action of toxicity and carcinogenicity in rodents. Food Chem Toxicol 80:206–214, doi:10.1016/j.fct.2015.03.023.

Lee HK, Kim TS, Kim CY, Kang IH, Kim MG, Jung KK, et al. 2012. Evaluation of *in vitro* screening system for estrogenicity: Comparison of stably transfected human estrogen receptor-α transcriptional activation (OECD TG455) assay and estrogen receptor (ER) binding assay. J Toxicol Sci 37:431–437, doi:10.2131/jts.37.431.

Lee IS, Kang HH, Kim UJ, Oh JE. 2015. Brominated flame retardants in Korean river sediments, including changes in polybrominated diphenyl ether concentrations between 2006 and 2009. Chemosphere 126:18–24, doi:10.1016/j.chemosphere.2015.01.004.

Li J, Ma M, Wang Z. 2010. *In vitro* profiling of endocrine disrupting effects of phenols. Toxicol In Vitro 24:201–207, doi:10.1016/j.tiv.2009.09.008.

Malkoske T, Tang Y, Xu W, Yu S, Wang H. 2016. A review of the environmental distribution, fate, and control of tetrabromobisphenol A released from sources. Sci Total Environ 569–570:1608–1617, doi:10.1016/j.scitotenv.2016.06.062.

Meerts IA, Letcher RJ, Hoving S, Marsh G, Bergman A, Lemmen JG, et al. 2001. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. Environ Health Perspect 109:399–407.

Miller BH, Takahashi JS. 2014. Central circadian control of female reproductive function. Front Endocrinol 5:1–8, doi:10.3389/fendo.2013.00195.

Miller D, Wheals BB, Beresford N, Sumpter JP. 2001. Estrogenic activity of phenolic additives determined by an in vitro yeast bioassay. Environ Health Perspect 109:133–138.

Million Women Study Collaborators. 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 362:419–427, doi:10.1016/S0140-6736(03)14065-2.

Million Women Study Collaborators. 2005. Endometrial cancer and hormone-replacement therapy in the Million Women Study. Lancet 365:1543–1551, doi:10.1016/S0140-6736(05)66455-0.

Million Women Study Collaborators. 2007. Ovarian cancer and hormone replacement therapy in the Million Women Study. Lancet 369:1703–1710, doi:10.1016/S0140-6736(07)60534-0.

Molina-Molina JM, Amaya E, Grimaldi M, Sáenz JM, Real M, Fernández MF, et al. 2013. *In vitro* study on the agonistic and antagonistic activities of bisphenol-S and

other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol Appl Pharmacol 272:127–136, doi:10.1016/j.taap.2013.05.015.

Muir C, Spironello-Vella E, Pisani N, deCatanzaro D. 2001. Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Horm Metab Res 33:653–658, doi:10.1055/s-2001-18692.

Nakao T, Akiyama E, Kakutani H, Mizuno A, Aozasa O, Akai Y, et al. 2015. Levels of tetrabromobisphenol A, tribromobisphenol A, dibromobisphenol A, monobromobisphenol A, and bisphenol A in Japanese breast milk. Chem Res Toxicol 28:722–728, doi:10.1021/tx500495j.

Ni HG, Zeng H. 2013. HBCD and TBBPA in particulate phase of indoor air in Shenzhen, China. Sci Total Environ 458–460:15–19, doi:10.1016/j.scitotenv.2013.04.003.

NIH (National Institutes of Health, National Center for Biotechnology Information). 2010. PubChem BioAssay Database; AID=893. Available: https://pubchem.ncbi.nlm.nih.gov/bioassay/893 [accessed 14 January 2017].

NTP (National Toxicology Program). 2014. Technical report on the toxicology studies of tetrabromobisphenol A (CAS NO. 79-94-7) in F344/NTac rats and B6C3F1/N mice and toxicology and carcinogenesis studies of tetrabromobisphenol A in Wistar Han [Crl:WI(Han)] rats and B6C3F1/N mice. Available: https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr587_508.pdf [accessed 15 October 2016].

Olsen CM, Meussen-Elholm ETM, Samuelsen M, Holme JA, Hongslo JK. 2003. Effects

of the environmental oestrogens bisphenol A, tetrachlorobisphenol A, tetrabromobisphenol A, 4-hydroxybiphenyl and 4,4'-dihydroxybiphenyl on oestrogen receptor binding, cell proliferation and regulation of oestrogen sensitive proteins. Pharmacol Toxicol 92:180–188.

Osimitz TG, Dourson ML, Hayes AW, Kacew S. 2014. Crystallographic analysis and mimicking of estradiol binding: Interpretation and speculation. Environ Health Perspect 122:A91.

Ozaki H, Sugihara K, Watanabe Y, Ohta S, Kitamura S. 2016. Cytochrome P450-inhibitory activity of parabens and phthalates used in consumer products. J Toxicol Sci 41:551–560.

Pollock T, deCatanzaro D. 2014. Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses. Reprod Toxicol 49:145–154, doi:10.1016/j.reprotox.2014.08.005.

Pollock T, Greville LJ, Tang B, deCatanzaro D. 2016. Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice. Reprod Toxicol 65:394–401, doi:10.1016/j.reprotox.2016.09.004.

Pollock T, Tang B, deCatanzaro D. 2014. Triclosan exacerbates the presence of ^{14}C-bisphenol A in tissues of female and male mice. Toxicol Appl Pharmacol 278:116–123, doi:10.1016/j.taap.2014.04.017.

Prusakiewicz JJ, Harville HM, Zhang Y, Ackermann C, Voorman RL. 2007. Parabens inhibit human skin estrogen sulfotransferase activity: Possible link to paraben estrogenic effects. Toxicology 232:248–256, doi:10.1016/j.tox.2007.01.010.

Queckenberg C, Meins J, Wachall B, Doroshyenko O, Tomalik-Scharte D, Bastian B, et al. 2010. Absorption, pharmacokinetics, and safety of triclosan after dermal administration. Antimicrob Agents Chemother 54:570–572, doi:10.1128/AAC.00615-09.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Riu A, Grimaldi M, le Maire A, Bey G, Phillips K, Boulahtouf A, et al. 2011a. Peroxisome proliferator-activated receptor γ is a target for halogenated analogs of bisphenol A. Environ Health Perspect 119:1227–1232, doi:10.1289/ehp.1003328.

Riu A, le Maire A, Grimaldi M, Audebert M, Hillenweck A, Bourguet W, et al. 2011b. Characterization of novel ligands of ERα, ERβ, and PPARγ: The case of halogenated bisphenol A and their conjugated metabolites. Toxicol Sci 122:372–382, doi:10.1093/toxsci/kfr132.

Rochester JR. 2013. Bisphenol A and human health: A review of the literature. Reprod Toxicol 42:132–155, doi:10.1016/j.reprotox.2013.08.008.

Sanders JM, Coulter SJ, Knudsen GA, Dunnick JK, Kissling GE, Birnbaum LS. 2016. Disruption of estrogen homeostasis as a mechanism for uterine toxicity in Wistar Han rats treated with tetrabromobisphenol A. Toxicol Appl Pharmacol 298:31–39, doi:10.1016/j.taap.2016.03.007.

Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, Keri RA. 2016. A review of the carcinogenic potential of bisphenol A. Reprod Toxicol 59:167–182, doi:10.1016/j.reprotox.2015.09.006.

Shaw SD, Harris JH, Berger ML, Subedi B, Kannan K. 2014. Brominated flame retardants and their replacements in food packaging and household products: Uses, human exposure, and health effects. In *Toxicants in Food Packaging and Household Plastics: Exposure and Health Risks to Consumers* (S. Snedeker, ed.), pp. 61–93, Springer, London.

Sieli PT, Jašarevic E, Warzak DA, Mao J, Ellersieck MR, Liao C, et al. 2011. Comparison of serum bisphenol A concentrations in mice exposed to bisphenol A through the diet versus oral bolus exposure. Environ Health Perspect 119:1260–1265.

Stoker TE, Gibson EK, Zorrilla LM. 2010. Triclosan exposure modulates estrogen-dependent responses in the female Wistar rat. Toxicol Sci 117:45–53, doi:10.1093/toxsci/kfq180.

Suzuki G, Tue NM, Malarvannan G, Sudaryanto A, Takahashi S, Tanabe S, et al. 2013. Similarities in the endocrine-disrupting potencies of indoor dust and flame retardants by using human osteosarcoma (U2OS) cell-based reporter gene assays. Environ Sci Technol 47:2898–2908, doi:10.1021/es304691a.

Thorpe JB, Burgess PS, Sadkowski M, deCatanzaro D. 2013. Estrogen-progesterone balance in the context of blastocyst implantation failure induced by predator stress. Psychoneuroendocrinology 38:3048–3056, doi:10.1016/j.psyneuen.2013.09.001.

Thorpe JB, Gould KE, Borman ED, deCatanzaro D. 2014. Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*). Horm Metab Res 46:211–218, doi:10.1055/s-0033-

1363958.

Wang J, Liu L, Wang J, Pan B, Fu X, Zhang G, et al. 2015a. Distribution of metals and brominated flame retardants (BFRs) in sediments, soils and plants from an informal e-waste dismantling site, South China. Environ Sci Pollut Res 22:1020–1033, doi:10.1007/s11356-014-3399-1.

Wang L-Q, Falany CN, James MO. 2004. Triclosan as a substrate and inhibitor of 3′-phosphoadenosine 5′-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. Pharmacology 32:1162–1169, doi:10.1124/dmd.104.000273.the.

Wang L-Q, James MO. 2007. Sulfonation of 17β-estradiol and inhibition of sulfotransferase activity by polychlorobiphenylols and celecoxib in channel catfish, *Ictalurus punctatus*. Aquat Toxicol 81:286–292, doi:10.1016/j.aquatox.2006.12.011.

Wang W, Abualnaja KO, Asimakopoulos AG, Covaci A, Gevao B, Johnson-Restrepo B, et al. 2015b. A comparative assessment of human exposure to tetrabromobisphenol A and eight bisphenols including bisphenol A via indoor dust ingestion in twelve countries. Environ Int 83:183–191, doi:10.1016/j.envint.2015.06.015.

Wikoff D, Thompson C, Perry C, White M, Borghoff S, Fitzgerald L, et al. 2015. Development of toxicity values and exposure estimates for tetrabromobisphenol A: Application in a margin of exposure assessment. J Appl Toxicol 35:1292–1308, doi:10.1002/jat.3132.

Wikoff DS, Rager JE, Haws LC, Borghoff SJ. 2016. A high dose mode of action for

tetrabromobisphenol A-induced uterine adenocarcinomas in Wistar Han rats: A critical evaluation of key events in an adverse outcome pathway framework. Regul Toxicol Pharmacol 77:143–159, doi:10.1016/j.yrtph.2016.01.018.

Wu Y, Chitranshi P, Loukotková L, Gamboa da Costa G, Beland FA, Zhang J, et al. 2016a. Cytochrome P450-mediated metabolism of triclosan attenuates its cytotoxicity in hepatic cells. Arch Toxicol 1–19, doi:10.1007/s00204-016-1893-6.

Wu Y, Li Y, Kang D, Wang J, Zhang Y, Du D, et al. 2016b. Tetrabromobisphenol A and heavy metal exposure via dust ingestion in an e-waste recycling region in Southeast China. Sci Total Environ 541:356–364, doi:10.1016/j.scitotenv.2015.09.038.

Xiong J, An T, Zhang C, Li G. 2015. Pollution profiles and risk assessment of PBDEs and phenolic brominated flame retardants in water environments within a typical electronic waste dismantling region. Environ Geochem Health 37:457–473, doi:10.1007/s10653-014-9658-8.

Yalcin EB, Kulkarni SR, Slitt AL, King R. 2016. Bisphenol A sulfonation is impaired in metabolic and liver disease. Toxicol Appl Pharmacol 292:75–84, doi:10.1016/j.taap.2015.12.009.

Yokota H, Iwano H, Endo M, Kobayashi T, Inoue H, Ikushiro S, et al. 1999. Glucuronidation of the environmental oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver. Biochem J 340 (Pt 2:405–409, doi:10.1042/0264-6021:3400405.

Zalko D, Prouillac C, Riu A, Perdu E, Dolo L, Jouanin I, et al. 2006. Biotransformation of the flame retardant tetrabromo-bisphenol A by human and rat sub-cellular liver

fractions. Chemosphere 64:318–327, doi:10.1016/j.chemosphere.2005.12.053.

Zalko D, Soto AM, Dolo L, Dorio C, Rathahao E, Debrauwer L, et al. 2003. Biotransformations of bisphenol A in a mammalian model: Answers and new questions raised by low-dose metabolic fate studies in pregnant CD1 mice. Environ Health Perspect 111:309–319, doi:10.1289/ehp.5603.

Zhu ZC, Chen SJ, Zheng J, Tian M, Feng AH, Luo XJ, et al. 2014. Occurrence of brominated flame retardants (BFRs), organochlorine pesticides (OCPs), and polychlorinated biphenyls (PCBs) in agricultural soils in a BFR-manufacturing region of North China. Sci Total Environ 481:47–54, doi:10.1016/j.scitotenv.2014.02.023.

Zhuang S, Zhang C, Liu W. 2014. Atomic insights into distinct hormonal activities of bisphenol A analogues toward PPARγ and ERα receptors. Chem Res Toxicol 27:1769–1779, doi:10.1021/tx500232b.

Ziv-Gal A, Flaws JA. 2016. Evidence for bisphenol A-induced female infertility: A review (2007–2016). Fertil Steril 106:827–856, doi:10.1016/j.fertnstert.2016.06.027.

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Chapter 7

A mixture of five endocrine-disrupting chemicals modulates concentrations of bisphenol A and estradiol in mice

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Authors' Contributions

Tyler Pollock: Experimental design, data collection, data analysis, manuscript writing, editing, and preparation.

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Abstract

Most people in developed countries are exposed to multiple endocrine-disrupting synthetic chemicals. We previously showed that a single dose of triclosan, tetrabromobisphenol A (TBBPA), butyl paraben, propyl paraben, or di(2-ethylhexyl) phthalate elevated concentrations of bisphenol A (BPA) in mice. Here we investigated whether concurrent exposure to lower doses of these five chemicals could modulate concentrations of bisphenol A (BPA) or the natural estrogen, 17β-estradiol (E2). CF1 mice were injected subcutaneously with 0.1 or 0.5 mg of one chemical, or a 0.5 mg mixture containing 0.1 mg of each of all five chemicals, then given a dietary supplement containing 50 μg/kg ^{14}C-BPA. The mixture elevated ^{14}C-BPA concentrations in the lungs, muscle, uterus, ovaries, kidney, and blood serum of female mice. When administered alone, triclosan and TBBPA elevated ^{14}C-BPA concentrations in the uterus, ovaries, and blood serum. In another experiment, CF1 mice were injected subcutaneously with the 0.5 mg mixture containing 0.1 mg of all five chemicals, then E2 was measured in urine 2–12 h later. The mixture elevated E2 at 8 h after injection in female mice. No treatments significantly altered concentrations of ^{14}C-BPA or E2 in male mice. These data show that these endocrine-disrupting chemicals interact *in vivo*, magnifying one another's effects, consistent with inhibition of enzymes that are critical for estrogen metabolism. These findings highlight the importance of considering exposure to multiple chemicals when assessing health outcomes and determining regulatory exposure limits.

Introduction

Recent evidence has shown that concurrent exposure to other common endocrine-disrupting chemicals (EDCs) can magnify the presence of bisphenol A (BPA, the monomer of polycarbonate plastics) in reproductive tissues and blood serum. Mice were exposed to varied doses of one of several EDCs then given 50 µg/kg ^{14}C-BPA. Administration of a single dose of 0.6–18 mg triclosan, a biocide found in soaps and other household products, elevated BPA concentrations in blood serum and tissues (Pollock et al. 2014). Similar effects were found with exposure to 1–27 mg tetrabromobisphenol A (TBBPA, a flame retardant found in consumer electronics) (Pollock et al. 2017a). Such effects have also been found following exposure to either 1–9 mg butyl paraben (BP) or 9 mg propyl paraben (PP), antimicrobial preservatives found in personal care products, pharmaceuticals, and foods (Pollock et al. 2017b). These effects were also observed after exposure to 3–18 mg di(2-ethylhexyl) phthalate (DEHP, a plasticizer found in flooring, medical devices, and food packaging) (Borman et al. 2017b). Lower doses of 0.33 mg triclosan or 0.33 mg TBBPA were ineffective on their own, but a concurrent dose of 0.33 mg triclosan + 0.33 mg TBBPA was sufficient to elevate concentrations of ^{14}C-BPA (Pollock et al. 2017a). Some of these substances can also alter concentrations of the powerful natural estrogen, 17β-estradiol (E2). Elevated urinary concentrations of E2 were observed in mice following a single dose of 1–2 mg triclosan (Pollock et al. 2016), 1 mg TBBPA (Pollock et al. 2017a), or 3 mg BP (Pollock et al. 2017b). Exposure to 0.6–2 mg triclosan prior to administering ^{3}H-E2 elevated radioactivity in the uterus, ovaries, and serum of females.

Disruption of estrogen homeostasis by EDCs could mediate the impact of these chemicals on blastocyst implantation in the uterus of inseminated females. Implantation is very sensitive to fluctuations in estrogen levels, being disrupted by very small elevations in E2 (deCatanzaro 2015; Gidley-Baird et al. 1986; Ma et al. 2003; Thorpe et al. 2013). Implantation can be disrupted in mice by daily doses of 18 mg triclosan (Crawford and deCatanzaro 2012), 36 mg DEHP (Borman et al. 2017a), or 6.75 mg BPA (Berger et al. 2007, 2008, 2010; Borman et al. 2015) on days 1 to 4 of gestation. Lower doses of 9 mg triclosan + 4 mg BPA or 27 mg DEHP + 3 mg BPA, which are ineffective on their own, disrupted implantation when given concurrently (Borman et al. 2017a; Crawford and deCatanzaro 2012). Estrogenic actions of common EDCs potentially have great importance for human health, considering that small elevations above optimal endogenous levels can be damaging to female fertility (Gidley-Baird et al. 1986; Ma et al. 2003; Thorpe et al. 2013) and are implicated in hormone-dependent cancers (Million Women Study Collaborators 2003, 2005, 2007).

These findings are consistent with evidence that these chemicals compete with each other and natural estrogens for access to metabolic enzymes. Enzymes involved in EDC and natural estrogen metabolism include sulfotransferase (SULT), UDP-glucuronosyltransferase (UGT), cytochrome p450 (CYP), and hydroxysteroid dehydrogenase (HSD) (Dumas and Diorio 2011). Given that BPA is an established EDC with estrogenic activity (Rochester 2013; Seachrist et al. 2016; Ziv-Gal and Flaws 2016), it is especially concerning that the potency of BPA in inducing biological effects may be greatly modulated by the presence of other EDCs. As humans are commonly exposed to

combinations of some or all of the EDCs mentioned above, it is important to consider their interactions in determining potential toxicological effects.

The current study was designed to test the influences of concurrent exposure to multiple EDCs, using lower doses that better reflect human exposures. We investigated the capacity of PP, DEHP, BP, triclosan, and TBBPA, either alone or as a mixture, to modulate concentrations of BPA and E2. We hypothesized that the effects of each chemical in the mixture would be additive, consistent with a mechanism whereby chemicals compete for access to metabolic enzymes. We tested this hypothesis by measuring the impact of PP, DEHP, BP, triclosan, or TBBPA injection, either alone or as a mixture, on concentrations of ^{14}C-BPA in female and male mice. We also hypothesized that the mixture of chemicals could elevate natural E2. We tested this hypothesis by measuring the impact of a mixture of PP, DEHP, BP, triclosan, and TBBPA injection on urinary E2 concentrations.

Methods

Animals and housing

Female (31.4 ± 3.3 g) and male (39.1 ± 3.1 g) CF-1 mice aged 2.5–4 months were obtained from Charles River (St. Constant, QC). Diestrous females were selected for use in experiments, as this is an easily detected point in the cycle where estrogen levels are relatively stable (Miller and Takahashi 2014). Females were identified from a colony of mice by vaginal cytology using published procedures (Byers et al. 2012). Animals were housed in polypropylene cages measuring 28×16×11 (l×w×h) cm with *ad libitum* access

to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University.

Chemicals and materials

Triclosan (CAS 3380-34-5, 5-chloro-2-[2,4-dichlorophenoxy]phenol, ≥97% purity), 3,3′,5,5′-TBBPA (CAS 79-94-7, 4,4′-isopropylidenebis[2,6-dibromophenol], ≥97% purity), BP (CAS 94-26-8, butyl 4-hydroxybenzoate, ≥99% purity), PP (CAS 94-13-3, propyl 4-hydroxybenzoate, ≥99% purity), DEHP (CAS 117-81-7, di(2-ethylhexyl) phthalate, ≥99% purity), E2 (CAS 50-28-2, ≥98% purity), and creatinine standards were obtained from Sigma-Aldrich, St. Louis, MO. ^{14}C-BPA ([ring-[^{14}C](U)]-BPA, in ethanol, 0.1 mCi/mL, 60 mCi/mmol) was obtained from Moravek Biochemicals, Brea, CA. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, 8 mL midi-vial scintillation vials were obtained from PerkinElmer, Waltham, MA. E2 antibodies and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA.

Experimental design and dosing

This research followed previously published procedures in this laboratory (Borman et al. 2017b; Pollock et al. 2014, 2016, 2017a, 2017b). Mice were weighed, individually housed, and each given a dietary supplement of 1 g peanut butter. About 14–16 h later, at the onset of darkness on the following day (8:00 AM), animals were

randomly assigned to treatment conditions involving a single subcutaneous (sc) injection of vehicle, one of the 5 chemicals, or a mixture of all 5 chemicals dissolved in 0.05 mL peanut oil. In Experiment 1, diestrous females and males received vehicle, 0.1 mg PP, 0.1 mg DEHP, 0.1 mg BP, 0.1 mg triclosan, 0.1 mg TBBPA, or a 0.5 mg mixture containing 0.1 mg of each of all 5 chemicals (n = 10 per dose). In Experiment 2, diestrous females and males received vehicle, 0.5 mg PP, 0.5 mg DEHP, 0.5 mg BP, 0.5 mg triclosan, 0.5 mg TBBPA, or a 0.5 mg mixture containing 0.1 mg of each of all 5 chemicals (n = 10 per dose). A dose of 0.1 mg corresponded to 3.4 ± 0.2 mg/kg in females and 2.6 ± 0.1 mg/kg in males, while a dose of 0.5 mg corresponded to 15.4 ± 1.6 mg/kg in females and 12.7 ± 1.2 mg/kg in males. At 30 min after injection, each animal was given a dietary supplement of 50 µg/kg ^{14}C-BPA in 0.2 g peanut butter. Food, water, and bedding were removed to prevent contamination of the ^{14}C-BPA treatment. At 1 h after ^{14}C-BPA administration, each animal was anesthetized with isoflurane and blood was collected via cardiac puncture. Each animal was perfused with 20 mL phosphate-buffered saline (PBS) and tissues were collected in pre-weighed scintillation vials. Tissue samples taken included the heart, lung, superficial adductor muscle from the hind leg, abdominal adipose, liver, and a cross-section of the kidney encompassing both the medulla and cortex. Female reproductive tissues taken included the whole uterus and both ovaries. Male reproductive tissues were one testis, one epididymis, one vesicular-coagulating (VC) gland, and one preputial gland. Vials were re-weighed following tissue collection to determine the sample wet mass.

In Experiment 3, mice were weighed and individually placed in a Plexiglas apparatus measuring 21×15×13 (l×w×h) cm with a wire-mesh grid floor raised approximately 1 cm above a Teflon-coated stainless-steel surface covered with wax paper. Animals acclimated to the novel cages for 3 days prior to the start of the experiment. At the onset of darkness on the fourth day, diestrous females and males were injected sc with vehicle or a 0.5 mg mixture containing 0.1 mg of each of all 5 chemicals dissolved in 0.05 mL peanut oil (n = 15 per dose). The 0.5 mg mixture corresponded to 16.1 ± 1.4 mg/kg in females and 13.2 ± 0.8 mg/kg in males. Urine was collected non-invasively at 2, 4, 6, 8, 10, and 12 h post-injection. All urine samples were placed into labeled vials and frozen at –20 °C at the time of collection.

Blood and tissue processing for liquid scintillation counting

Blood and tissue samples were processed for liquid scintillation counting following previously published procedures (deCatanzaro et al. 2014; deCatanzaro and Pollock 2016; Greville et al. 2017). Blood samples were centrifuged at 1,500 *g* for 10 min and 10 µL serum was added to a scintillation vial containing 5 mL Ultima Gold. Tissue samples were solubilized by adding 1 mL SOLVABLE to each vial and placing vials in a 50 °C water bath for 4–5 h until completely dissolved. Following the addition of 5 mL Ultima Gold, vials were agitated to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) for 5 min to eliminate noise in the form of heat and luminescence. Radioactivity was then measured for 5 min per vial. The amount of radioactivity per sample, in disintegrations per minute (dpm), was automatically

calculated via Quanta-Smart software by subtracting background radiation, which is continually monitored by the scintillation counter. Frequent cleaning and monitoring of all work surfaces and equipment ensured that contamination of samples did not occur. The final dpm measures were normalized to the weight of the sample wet mass and reported as equivalent ng BPA/g tissue or ng BPA/ml serum.

Measurement of urinary E2

Full procedures and validations for enzyme immunoassays for mouse urine were previously reported (Muir et al. 2001). Cross-reactivities for anti-E2 are: E2 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids <0.1%. Urinary E2 levels were considered with and without adjustment for urinary creatinine, which corrects for differential hydration and urinary concentration among animals, and reported as ng E2/mg creatinine and ng E2/ml urine respectively.

Statistical analyses

All analyses were performed using the R software environment (R Core Team 2017), with a comparison-wise error rate of $\alpha < 0.05$ for all tests. Differences among treatments in Experiments 1 and 2 were analyzed by univariate analysis of variance (ANOVA) for each tissue, using false discovery rate adjustments to correct for the number of tissues (Benjamini and Hochberg 1995). Significant effects in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons. Differences between urinary E2 concentrations of animals in Experiment 3 were analyzed by factorial

ANOVA comparing the effects of treatment and collection time-point (repeated measures), followed by Newman-Keuls comparisons at each time-point.

Results

Experiment 1: Measurement of ^{14}C-BPA in mice given 0.1 mg of each chemical

Pre-treatment with the mixture elevated concentrations of ^{14}C-BPA in serum and certain tissues of females (Figure 1), but not of males (Figure 2). Concentrations of ^{14}C-BPA in the liver and kidney are reported in Table 1. Comparisons were made among the seven treatments for each of nine tissues in females. ANOVA using false discovery rate adjustment produced significant effects of treatment for the lung, $F(6,63)=6.21$, $p<0.001$; muscle, $F(6,63)=5.07$, $p<0.001$; uterus, $F(6,63)=6.26$, $p<0.001$; ovaries, $F(6,63)=2.89$, $p=0.023$; kidney, $F(6,63)=4.13$, $p=0.002$; and serum, $F(6,63)=8.45$, $p<0.001$. Multiple comparisons revealed that the mixture treatment differed from all other treatments for the lung, muscle, and serum. The mixture treatment differed from all treatments except TBBPA for the uterus, and the TBBPA treatment differed from the vehicle treatment for the uterus. The mixture treatment also differed from the vehicle and BP treatments for the ovaries and from the vehicle treatment for the kidney. Comparable effects were not observed in males. Comparisons among the seven treatments for each of eleven tissues in males showed no statistically significant differences.

Figure 1 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females in Experiment 1. Animals received sc injection of vehicle, 0.1 mg PP, 0.1 mg DEHP, 0.1 mg BP, 0.1 mg triclosan, 0.1 mg TBBPA, or a 0.5 mg mixture containing 0.1 mg of all five chemicals followed by dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose). Significant differences among treatments within each tissue are denoted by unique letters.

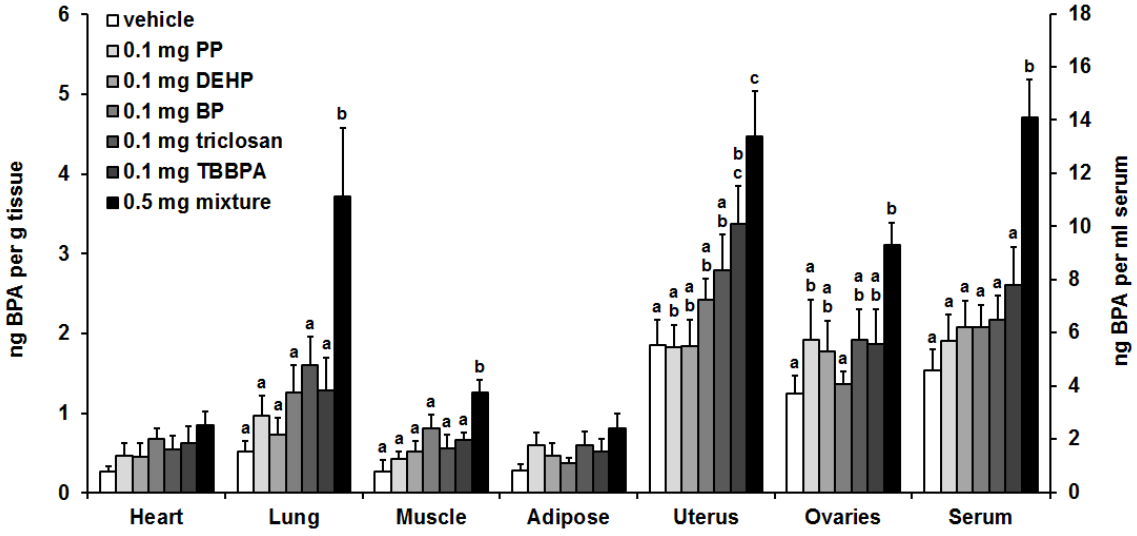


Figure 2 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, testes, epididymides, VC glands, preputial glands, and serum of males in Experiment 1. Animals received sc injection of vehicle, 0.1 mg PP, 0.1 mg DEHP, 0.1 mg BP, 0.1 mg triclosan, 0.1 mg TBBPA, or a 0.5 mg mixture containing 0.1 mg of all five chemicals followed by dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose).

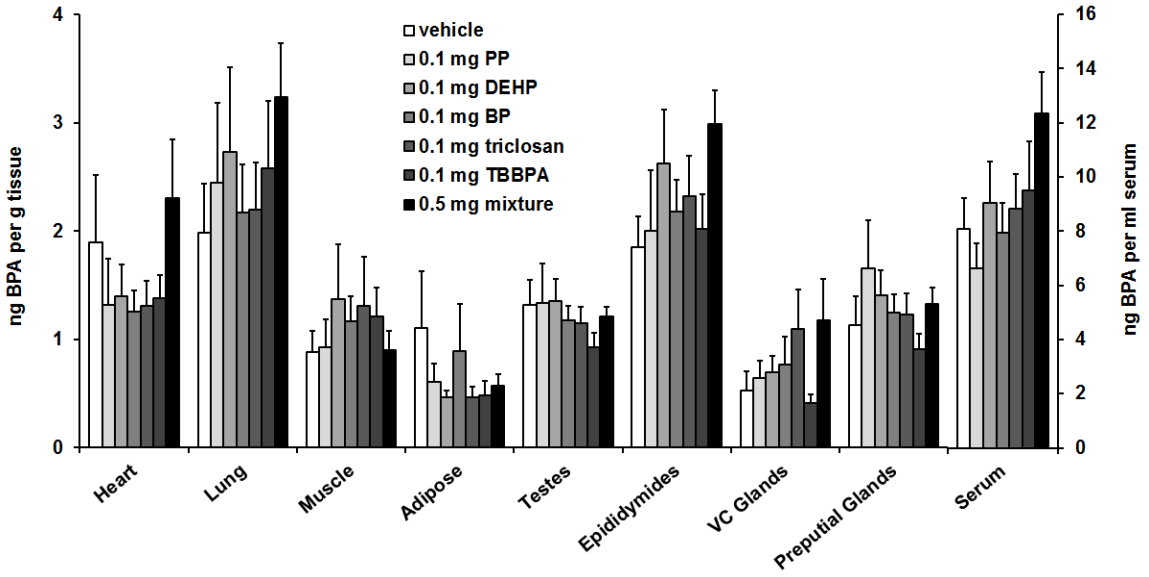


Table 1 Mean (± SE) concentration of ^{14}C-BPA in the liver and kidney of diestrous females and males following sc injection of PP, DEHP, BP, triclosan, TBBPA, or a mixture of all five chemicals and subsequent dietary administration of 50 µg/kg ^{14}C-BPA. Difference from vehicle treatment in the same tissue: + $p < 0.001$

	Chemical	Dose (mg)	Liver (ng BPA/g)	Kidney (ng BPA/g)
Experiment 1				
Females	vehicle	0.0	29.6 ± 2.2	15.1 ± 1.5
	PP	0.1	57.8 ± 14.2	26.9 ± 2.3
	DEHP	0.1	49.4 ± 7.3	26.0 ± 3.7
	BP	0.1	64.9 ± 17.5	24.6 ± 4.5
	triclosan	0.1	38.3 ± 3.4	21.3 ± 2.6
	TBBPA	0.1	52.3 ± 11.5	27.3 ± 3.3
	mixture	5 × 0.1	55.3 ± 6.7	37.4 ± 4.2 $^{+}$
Males	vehicle	0.0	45.0 ± 3.5	81.3 ± 11.1
	PP	0.1	46.3 ± 5.7	101.7 ± 27.6
	DEHP	0.1	44.3 ± 6.7	92 ± 15.7
	BP	0.1	27.8 ± 2.1	89.9 ± 12.7
	triclosan	0.1	46.0 ± 7.4	79.8 ± 8.8
	TBBPA	0.1	44.6 ± 6.7	79.8 ± 9.0
	mixture	5 × 0.1	43.7 ± 3.0	115.5 ± 9.7
Experiment 2				
Females	vehicle	0.0	36.8 ± 4.3	24.3 ± 5.1
	PP	0.5	39.1 ± 7.1	20.1 ± 3.5
	DEHP	0.5	39.8 ± 7.8	30.1 ± 6.7
	BP	0.5	49.6 ± 8.7	22.6 ± 2.8
	triclosan	0.5	65.7 ± 9.9	37.1 ± 5.6
	TBBPA	0.5	61.6 ± 11.0	51.4 ± 14.0
	mixture	5 × 0.1	52.9 ± 4.1	32.7 ± 3.4
Males	Vehicle	0.0	40.4 ± 3.4	67.0 ± 8.7
	PP	0.5	52.3 ± 6.7	79.8 ± 16.6
	DEHP	0.5	47.0 ± 3.5	76.3 ± 11.6
	BP	0.5	36.8 ± 4.7	71.8 ± 9.0
	Triclosan	0.5	59.1 ± 8.5	125.8 ± 15.1
	TBBPA	0.5	47.8 ± 9.0	79.6 ± 18.9
	Mixture	5 × 0.1	46.5 ± 4.2	94.9 ± 9.8

Experiment 2: Measurement of ^{14}C-BPA in mice given 0.5 mg of each chemical

Radioactivity in diestrous females (Figure 3) was elevated in serum and reproductive tissues by pre-treatment with triclosan, TBBPA, or the mixture. Comparisons among the seven treatments for each of nine tissues in females showed significant effects of treatment for the uterus, $F(6,63)=7.24$, $p<0.001$; ovaries, $F(6,63)=5.94$, $p<0.001$; and serum, $F(6,63)=7.41$, $p<0.001$. Multiple comparisons revealed that the mixture, triclosan, and TBBPA treatments differed from the vehicle, PP, DEHP, and BP treatments for the uterus. The mixture treatment differed from the vehicle, PP, and DEHP treatments for the serum. The triclosan and TBBPA treatments differed from the vehicle, PP, DEHP, and BP treatments for the ovaries and serum. Comparable effects were once again not seen in males (Figure 4). Comparisons among the seven treatments for each of eleven tissues in males showed no statistically significant differences.

Figure 3 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, uterus, ovaries, and serum of diestrous females in Experiment 2. Animals received sc injection of vehicle, 0.5 mg PP, 0.5 mg DEHP, 0.5 mg BP, 0.5 mg triclosan, 0.5 mg TBBPA, or a 0.5 mg mixture containing 0.1 mg of all five chemicals followed by dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose). Significant differences among treatments within each tissue are denoted by unique letters.

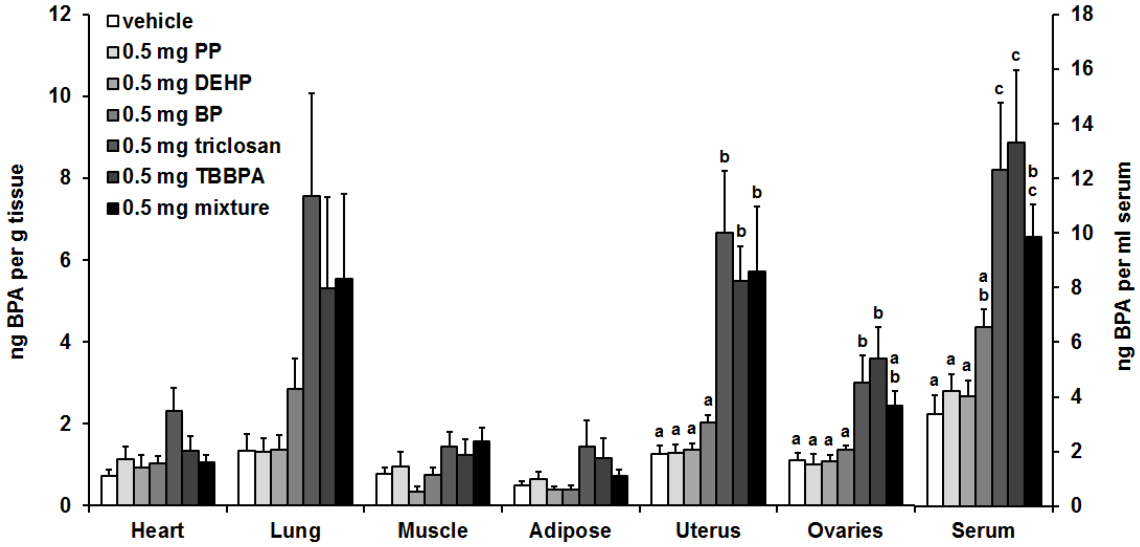
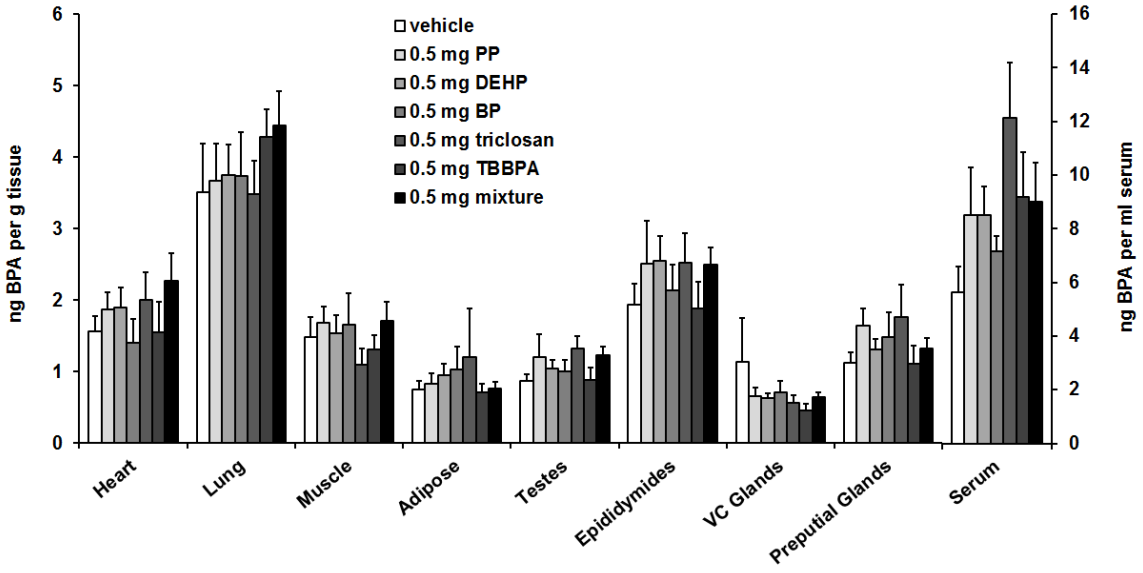


Figure 4 Mean (+SE) concentration of ^{14}C-BPA in the heart, lung, muscle, adipose, testes, epididymides, VC glands, preputial glands, and serum of males in Experiment 2. Animals received sc injection of vehicle, 0.5 mg PP, 0.5 mg DEHP, 0.5 mg BP, 0.5 mg triclosan, 0.5 mg TBBPA, or a 0.5 mg mixture containing 0.1 mg of all five chemicals followed by dietary administration of 50 µg/kg ^{14}C-BPA (n=10 per dose).



Experiment 3: Measurement of urinary E2 in mice

Concentrations of E2 are reported for uncorrected (ng E2/ml urine) and corrected (ng E2/mg creatinine) measures. In females (Figure 5), there were no statistically significant main effects or interaction for uncorrected measures; however, corrected measures showed a significant main effect of treatment, $F(1,26)=4.58$, $p=0.042$. Multiple comparisons revealed that the vehicle-treated females differed from the mixture-treated ones at 8 h after injection for the corrected measures. In males (Figure 6), ANOVA showed a significant main effect of collection time-point on uncorrected measures, $F(5,70)=9.48$, $p<0.001$, and on corrected measures, $F(5,70)=12.05$, $p<0.001$. For both uncorrected and corrected measures, main effect of treatment and interaction did not reach statistical significance.

Figure 5 Mean (+SE) concentration of urinary E2, expressed as ng E2/ml urine and ng E2/mg creatinine, of diestrous females in Experiment 3. Females received sc injection of vehicle or a 0.5 mg mixture containing 0.1 mg of all five chemicals (n=15 per dose). Significant difference from vehicle treatment at the same time-point: * $p < 0.05$

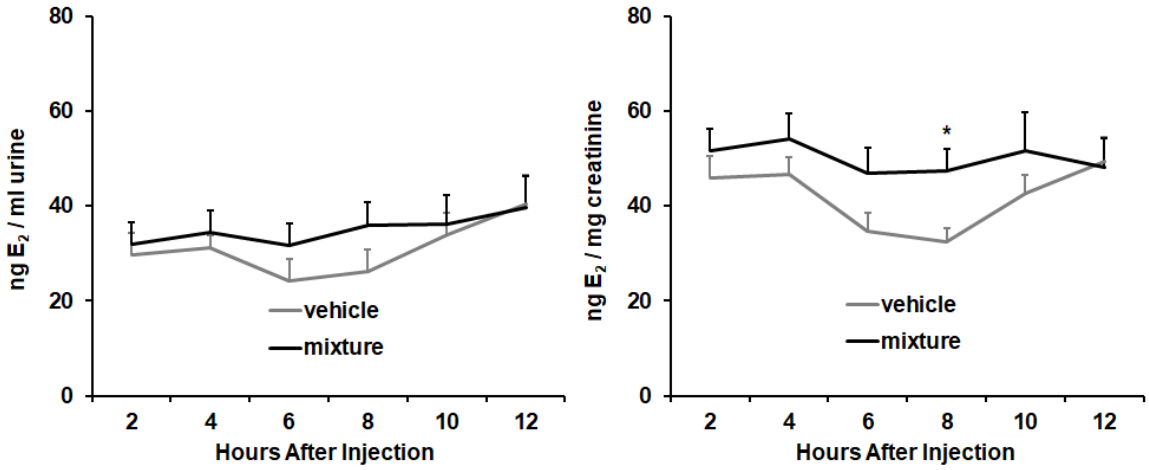
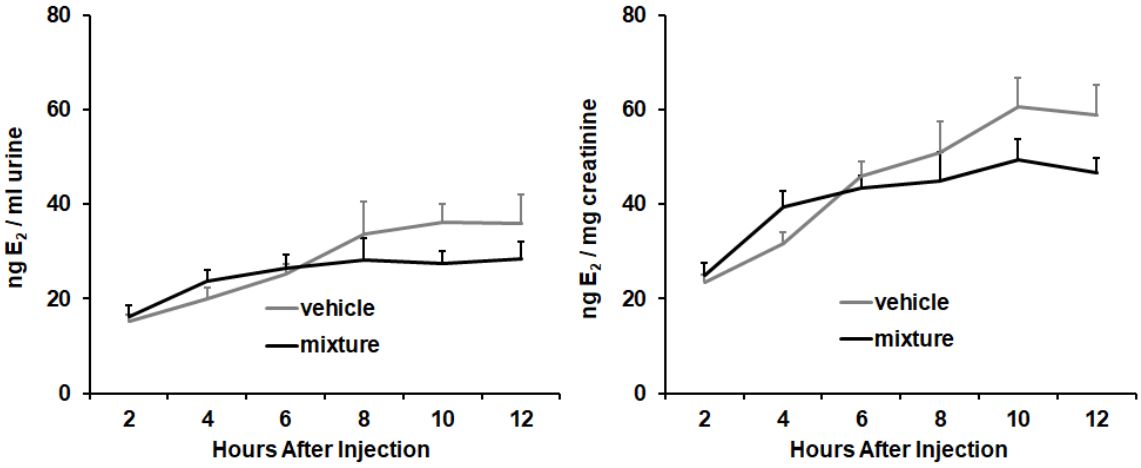


Figure 6 Mean (+SE) concentration of urinary E2, expressed as ng E2/ml urine and ng E2/mg creatinine, of males in Experiment 3. Males received sc injection of vehicle or a 0.5 mg mixture containing 0.1 mg of all five chemicals (n=15 per dose).



Discussion

These data demonstrate *in vivo* modulation of BPA concentrations by a mixture of five EDCs. When animals were given a dietary supplement containing ^{14}C-BPA, pre-treatment with a 0.5 mg mixture containing 0.1 mg of each of PP, DEHP, BP, triclosan, and TBBPA increased radioactivity in blood serum, reproductive organs, and other tissues of female mice. These data show a clear *in vivo* interaction among these chemicals, as most were ineffective when given alone. When each chemical was given alone at the 0.1 mg dose, only TBBPA elevated radioactivity significantly, and only in the uterus. At the 0.5 mg dose, triclosan alone or TBBPA alone elevated radioactivity in the uterus, ovaries, and serum. Effects of triclosan, TBBPA, and especially the mixture were pronounced in reproductive organs that contain high ER expression (Couse et al. 1997; Kuiper et al. 1997). Concentrations of ^{14}C-BPA were greatest in the liver and kidney, consistent with previous studies of the distribution of 0.5–100,000 µg/kg BPA (Kim et al. 2004; Kurebayashi et al. 2005; Pollock and deCatanzaro 2014). These organs are involved in the metabolism and excretion of ingested BPA and do not necessarily reflect tissue deposition.

The findings of Experiments 1 and 2 are consistent with previous studies of the effects of PP, DEHP, BP, triclosan, or TBBPA on the toxicokinetics of BPA (Borman et al. 2017b; Pollock et al. 2014, 2017a, 2017b). However, much higher doses, ranging from 0.6 to 27 mg, were required to elevate BPA concentrations significantly when animals were pre-treated with a single chemical. Here, the data indicate additive effects of lower doses of these five chemicals that collectively magnify concentrations of BPA, and they

suggest common mechanisms of action. The most plausible mechanisms involve competition among these chemicals for access to metabolic enzymes. BPA is primarily metabolized by phase II conjugative enzymes, including UGT and SULT (Hanioka et al. 2008; Pritchett et al. 2002; Zalko et al. 2003). BPA monoglucuronide is the major metabolite of BPA and results from interaction with UGT (Hanioka et al. 2008; Kurebayashi et al. 2010; Zalko et al. 2003). Other metabolites include the monosulfate conjugate produced by SULT (Yalcin et al. 2016), as well as various diconjugates (Inoue et al. 2016; Yalcin et al. 2016; Zalko et al. 2003). Production of glucuronide and sulfate conjugates has been shown for TBBPA (Borghoff et al. 2016), triclosan (Fang et al. 2014), and parabens (Moos et al. 2016). Glucuronide (Frederiksen et al. 2007; Ito et al. 2005; Silva et al. 2003), but not sulfate (Witzmann et al. 1996), conjugates have been shown for MEHP, a major metabolite of DEHP (Hanioka et al. 2012, 2016). UGT and SULT activities towards other substrates are inhibited by triclosan (James et al. 2010, 2015; Wang et al. 2004), parabens (Abbas et al. 2010; Prusakiewicz et al. 2007), and TBBPA (Gosavi et al. 2013; Hamers et al. 2006; Harju et al. 2007; Kester et al. 2002). Interestingly, the potency with which each chemical can inhibit phase II metabolic enzyme activity generally corresponds to the capacity with which these chemicals can modulate BPA concentrations in serum and reproductive organs of female mice, in the order TBBPA ≈ triclosan > BP > DEHP ≈ PP.

Urinary E2 concentrations of female mice were also elevated by the mixture of five EDCs. We measured E2 in urine because it can be collected non-invasively at multiple time-points and generally reflects systemic estrogen trends (deCatanzaro et al.

2003, 2004; Muir et al. 2001; Thorpe et al. 2014). Data of Experiment 3 are consistent with previous studies showing that higher doses (1–3 mg) of BP, triclosan, or TBBPA each increased E2 in urine at 6–12 h after injection (Pollock et al. 2016, 2017a, 2017b). Here, the mixture containing lower doses (0.1 mg) of all 5 chemicals elevated E2 in urine at 8 h after injection. These data are consistent with a mechanism of action whereby EDCs can disrupt estrogen homeostasis by blocking access to metabolic enzymes (Lai et al. 2015; Sanders et al. 2016; Wikoff et al. 2016) and/or increasing E2 biosynthesis (Honkisz and Wójtowicz 2015). In addition to phase II conjugative enzymes described above, phase I enzymes, including cytochrome p450 (CYP) and hydroxysteroid dehydrogenases (HSD), are important for estrogen regulation (Dumas and Diorio 2011). Triclosan (Wu et al. 2017), parabens (Ozaki et al. 2016), MEHP (Choi et al. 2012, 2013), and TBBPA (Ames 2013) are substrates and inhibitors of CYP. TBBPA can also inhibit 17β-HSD4 activity (NIH 2010). Given the 8-h latency between treatment with the chemical mixture and elevation of E2 concentrations, this effect could also be explained by increased E2 biosynthesis rather than, or in addition to, reduced E2 metabolism. Triclosan (Honkisz et al. 2012) and TBBPA (Honkisz and Wójtowicz 2015) upregulate aromatase protein expression and increase E2 secretion in JEG-3 human choriocarcinoma cells *in vitro*. Parabens upregulate aromatase gene and protein expression and increase E2 secretion in MCF-7 human breast cancer cells *in vitro* (Wróbel and Gregoraszczuk 2013). The combined effects of triclosan, TBBPA, PP, and BP on E2 secretion could elevate E2 concentrations *in vivo*.

The five chemicals incorporated into the mixture were chosen based on their inclusion in the EPA's Endocrine Disruptor Screening Program (EDSP) (U.S. EPA 2012) and their ubiquitous presence in consumer products. As a result, these chemicals are present at detectable levels in most people. Based on the 2011–2012 U.S. National Health and Nutrition Examination Survey (NHANES), 72% of human urine samples contain detectable triclosan concentrations ranging from 2.3 to 3,830 μg/L with a mean of 12.5 μg/L (Han et al. 2016). Based on the 2005–2006 U.S. NHANES, PP was detected in 92.7% of human urine samples with concentrations ranging from 0.2–7,210 μg/L and a mean of 7.9 μg/L (Calafat et al. 2010). Consistent with less prevalent use in consumer products (Błędzka et al. 2014), BP was detected in 47% of human urine samples with concentrations ranging from 0.2–1,240 μg/L and a mean of 0.90 μg/L in females (Calafat et al. 2010). Whereas DEHP was not assessed, several of its metabolites were included in the 2009–2010 U.S. NHANES. MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) were detected in 56, 98, 96, and 100% of human urine samples with means of 2.0, 13.8, 8.7, and 20.4 μg/L, respectively (Zota et al. 2014). Although TBBPA is not assessed in the U.S. NHANES, a recent study of 140 adults in China found that TBBPA is present in 89% of urine samples with concentrations up to 88.4 μg/g creatinine and a mean of 0.2 μg/g creatinine (Ho et al. 2017).

The ability of EDCs to disrupt natural estrogen homeostasis is of importance considering that slight but persistent elevations in E2 can contribute to adverse reproductive and other health outcomes in mammals. Increased E2 can lead to pregnancy

failure by disrupting intrauterine blastocyst implantation (deCatanzaro 2015; Gidley-Baird et al. 1986; Ma et al. 2003; Thorpe et al. 2013). In humans, higher serum E2 in the first trimester is associated with increased risk of low birth weight (Hu et al. 2014). Heightened estrogen activity from hormone-replacement therapy is associated with increased risk of breast, endometrial, and ovarian cancers (Million Women Study Collaborators 2003, 2005, 2007). Our findings demonstrate the importance of studies of multiple toxicants to elucidate the adverse health and reproductive outcomes of chemical mixtures. Exposure to numerous chemicals could potentially have adverse effects at doses that are much closer to human exposure than previously assumed. Thus, studies of multiple EDCs should be considered when conducting quantitative risk assessment and determining regulatory exposure limits.

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References

Abbas S, Greige-Gerges H, Karam N, Piet M-H, Netter P, Magdalou J. 2010. Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man. Drug Metab. Pharmacokinet. 25:568–577; doi:10.2133/dmpk.DMPK-10-RG-013.

Ames B. 2013. Tetrabrominated bisphenol A inhibition studies on six cytochrome P450 enzymes in human liver microsomes. California State University (dissertation).

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57: 289–300.

Berger RG, Foster WG, deCatanzaro D. 2010. Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. Reprod. Toxicol. 30:393–400; doi:10.1016/j.reprotox.2010.06.006.

Berger RG, Hancock T, deCatanzaro D. 2007. Influence of oral and subcutaneous bisphenol-A on intrauterine implantation of fertilized ova in inseminated female mice. Reprod. Toxicol. 23:138–144; doi:10.1016/j.reprotox.2006.09.005.

Berger RG, Shaw J, deCatanzaro D. 2008. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. Reprod. Toxicol. 26:94–99; doi:10.1016/j.reprotox.2008.06.007.

Błędzka D, Gromadzińska J, Wąsowicz W. 2014. Parabens. From environmental studies to human health. Environ. Int. 67:27–42; doi:10.1016/j.envint.2014.02.007.

Borghoff SJ, Wikoff D, Harvey S, Haws L. 2016. Dose- and time-dependent changes in

tissue levels of tetrabromobisphenol A (TBBPA) and its sulfate and glucuronide conjugates following repeated administration to female Wistar Han Rats. Toxicol. Reports 3:190–201; doi:10.1016/j.toxrep.2016.01.007.

Borman ED, Foster WG, deCatanzaro D. 2017a. Concurrent administration of diethylhexyl phthalate reduces the threshold dose at which bisphenol A disrupts blastocyst implantation and cadherins in mice. Environ. Toxicol. Pharmacol. 49:105–111; doi:10.1016/j.etap.2016.12.003.

Borman ED, Foster WG, Greenacre MKE, Muir CC, deCatanzaro D. 2015. Stress lowers the threshold dose at which bisphenol A disrupts blastocyst implantation, in conjunction with decreased uterine closure and e-cadherin. Chem. Biol. Interact. 237:87–95; doi:10.1016/j.cbi.2015.05.012.

Borman ED, Vecchi N, Pollock T, deCatanzaro D. 2017b. Diethylhexyl phthalate magnifies deposition of ^{14}C-bisphenol A in reproductive tissues of mice. J. Appl. Toxicol. In press; doi:10.1002/jat.3484.

Byers SL, Wiles M V., Dunn SL, Taft RA. 2012. Mouse estrous cycle identification tool and images. PLoS One 7:e35538; doi:10.1371/journal.pone.0035538.

Calafat AM, Ye X, Wong LY, Bishop AM, Needham LL. 2010. Urinary concentrations of four parabens in the U.S. Population: NHANES 2005–2006. Environ. Health Perspect. 118:679–685; doi:10.1289/ehp.0901560.

Choi K, Joo H, Campbell JL, Andersen ME, Clewell HJ. 2013. *In vitro* intestinal and hepatic metabolism of di(2-ethylhexyl) phthalate (DEHP) in human and rat. Toxicol. In Vitro 27:1451–1457; doi:10.1016/j.tiv.2013.03.012.

Choi K, Joo H, Campbell JL, Clewell RA, Andersen ME, Clewell HJ. 2012. *In vitro* metabolism of di(2-ethylhexyl) phthalate (DEHP) by various tissues and cytochrome P450s of human and rat. Toxicol. In Vitro 26:315–322; doi:10.1016/j.tiv.2011.12.002.

Couse JF, Lindzey J, Grandien K, Gustafsson J, Korach KS. 1997. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 138:4613–4621; doi:10.1210/en.138.11.4613.

Crawford BR, deCatanzaro D. 2012. Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A. Reprod. Toxicol. 34:607–613; doi:10.1016/j.reprotox.2012.09.008.

deCatanzaro D. 2015. Sex steroids as pheromones in mammals: The exceptional role of estradiol. Horm. Behav. 68:103–116; doi:10.1016/j.yhbeh.2014.08.003.

deCatanzaro D, Muir C, Beaton EA, Jetha M. 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69:687–696; doi:10.1016/j.steroids.2004.07.002.

deCatanzaro D, Muir C, Beaton E, Jetha M, Nadella K. 2003. Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 126:407–414; doi:10.1530/reprod/126.3.407.

deCatanzaro D, Pollock T. 2016. Absorption and distribution of estradiol from male seminal emissions during mating. J. Endocrinol. 231:245–257; doi:10.1530/JOE-16-

0247.

deCatanzaro D, Pollock T, Greville LJ, Faure PA. 2014. Estradiol transfer from male big brown bats (*Eptesicus fuscus*) to the reproductive and brain tissues of cohabiting females, and its action as a pheromone. Gen. Comp. Endocrinol. 208:126–133; doi:10.1016/j.ygcen.2014.09.011.

Dumas I, Diorio C. 2011. Estrogen pathway polymorphisms and mammographic density. Anticancer Res. 31: 4369–4386.

Fang J-L, Vanlandingham M, da Costa GG, Beland FA. 2014. Absorption and metabolism of triclosan after application to the skin of B6C3F1 mice. Environ. Toxicol. 31: 609–623.

Frederiksen H, Skakkebæk NE, Andersson AM. 2007. Metabolism of phthalates in humans. Mol. Nutr. Food Res. 51:899–911; doi:10.1002/mnfr.200600243.

Gidley-Baird AA, O'Neill C, Sinosich MJ, Porter RN, Pike IL, Saunders DM. 1986. Failure of implantation in human in vitro fertilization and embryo transfer patients: the effects of altered progesterone/estrogen ratios in humans and mice. Fertil. Steril. 45:69–74; doi:10.1016/S0015-0282(16)49099-0.

Gosavi RA, Knudsen GA, Birnbaum LS, Pedersen LC. 2013. Mimicking of estradiol binding by flame retardants and their metabolites: A crystallographic analysis. Environ. Health Perspect. 121:1194–1199; doi:10.1289/ehp.1306902.

Greville LJ, Pollock T, Salter JC, Faure PA, deCatanzaro D. 2017. Progesterone transfer among cohabitating female big brown bats (*Eptesicus fuscus*). Gen. Comp. Endocrinol. 247:199–204; doi:10.1016/j.ygcen.2017.02.003.

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MHA, Andersson PL, et al. 2006. *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol. Sci. 92:157–173; doi:10.1093/toxsci/kfj187.

Han C, Lim YH, Hong YC. 2016. Ten-year trends in urinary concentrations of triclosan and benzophenone-3 in the general U.S. population from 2003 to 2012. Environ. Pollut. 208:803–810; doi:10.1016/j.envpol.2015.11.002.

Hanioka N, Isobe T, Kinashi Y, Tanaka-Kagawa T, Jinno H. 2016. Hepatic and intestinal glucuronidation of mono(2-ethylhexyl) phthalate, an active metabolite of di(2-ethylhexyl) phthalate, in humans, dogs, rats, and mice: An *in vitro* analysis using microsomal fractions. Arch. Toxicol. 90:1651–1657; doi:10.1007/s00204-015-1619-1.

Hanioka N, Naito T, Narimatsu S. 2008. Human UDP-glucuronosyltransferase isoforms involved in bisphenol A glucuronidation. Chemosphere 74:33–36; doi:10.1016/j.chemosphere.2008.09.053.

Hanioka N, Takahara Y, Takahara Y, Tanaka-Kagawa T, Jinno H, Narimatsu S. 2012. Hydrolysis of di-*n*-butyl phthalate, butylbenzyl phthalate and di(2-ethylhexyl) phthalate in human liver microsomes. Chemosphere 89:1112–1117; doi:10.1016/j.chemosphere.2012.05.095.

Harju M, Hamers T, Kamstra JH, Sonneveld E, Boon JP, Tysklind M, et al. 2007. Quantitative structure–activity relationship modeling on *in vitro* endocrine effects and metabolic stability involving 26 selected brominated flame retardants. Environ. Toxicol. Chem. 26:816–826; doi:10.1897/06-308R.1.

Ho KL, Yuen KK, Yau MS, Murphy MB, Wan Y, Fong BMW, et al. 2017. Glucuronide and sulfate conjugates of tetrabromobisphenol A (TBBPA): Chemical synthesis and correlation between their urinary levels and plasma TBBPA content in voluntary human donors. Environ. Int. 98:46–53; doi:10.1016/j.envint.2016.09.018.

Honkisz E, Wójtowicz AK. 2015. Modulation of estradiol synthesis and aromatase activity in human choriocarcinoma JEG-3 cells exposed to tetrabromobisphenol A. Toxicol. In Vitro 29:44–50; doi:10.1016/j.tiv.2014.09.003.

Honkisz E, Zieba-Przybylska D, Wojtowicz AK. 2012. The effect of triclosan on hormone secretion and viability of human choriocarcinoma JEG-3 cells. Reprod. Toxicol. 34:385–392; doi:10.1016/j.reprotox.2012.05.094.

Hu XL, Feng C, Lin XH, Zhong ZX, Zhu YM, Lv PP, et al. 2014. High maternal serum estradiol environment in the first trimester is associated with the increased risk of small-for-gestational-age birth. J. Clin. Endocrinol. Metab. 99:2217–2224; doi:10.1210/jc.2013-3362.

Inoue H, Kemanai S, Sano C, Kato S, Yokota H, Iwano H. 2016. Bisphenol A glucuronide/sulfate diconjugate in perfused liver of rats. J. Vet. Med. Sci. 78:733–737; doi:10.1292/jvms.15-0573.

Ito Y, Yokota H, Wang R, Yamanoshita O, Ichihara G, Wang H, et al. 2005. Species differences in the metabolism of di(2-ethylhexyl) phthalate (DEHP) in several organs of mice, rats, and marmosets. Arch. Toxicol. 79:147–154; doi:10.1007/s00204-004-0615-7.

James MO, Ambadapadi S, Falany C. 2015. Triclosan inhibits the activity of expressed

human sulfotransferases (SULTs) towards their diagnostic substrates. FASEB J. 29: 622 (Abstract).

James MO, Li W, Summerlot DP, Rowland-Faux L, Wood CE. 2010. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ. Int. 36:942–949; doi:10.1016/j.envint.2009.02.004.

Kester MHA, Bulduk S, van Toor H, Tibboel D, Meinl W, Glatt H, et al. 2002. Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J. Clin. Endocrinol. Metab. 87:1142–1150; doi:10.1210/jcem.87.3.8311.

Kim CS, Sapienza PP, Ross IA, Johnson W, Luu HMD, Hutter JC. 2004. Distribution of bisphenol A in the neuroendocrine organs of female rats. Toxicol. Ind. Health 20:41–50; doi:10.1191/0748233704th186oa.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138: 863–870.

Kurebayashi H, Nagatsuka SI, Nemoto H, Noguchi H, Ohno Y. 2005. Disposition of low doses of ^{14}C-bisphenol A in male, female, pregnant, fetal, and neonatal rats. Arch. Toxicol. 79:243–252; doi:10.1007/s00204-004-0628-2.

Kurebayashi H, Okudaira K, Ohno Y. 2010. Species difference of metabolic clearance of bisphenol A using cryopreserved hepatocytes from rats, monkeys and humans. Toxicol. Lett. 198:210–215; doi:10.1016/j.toxlet.2010.06.017.

Lai DY, Kacew S, Dekant W. 2015. Tetrabromobisphenol A (TBBPA): Possible modes of action of toxicity and carcinogenicity in rodents. Food Chem. Toxicol. 80:206–214; doi:10.1016/j.fct.2015.03.023.

Ma W, Song H, Das SK, Paria BC, Dey SK. 2003. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Proc. Natl. Acad. Sci. 100:2963–2968; doi:10.1073/pnas.0530162100.

Miller BH, Takahashi JS. 2014. Central circadian control of female reproductive function. Front. Endocrinol. (Lausanne). 4:1–8; doi:10.3389/fendo.2013.00195.

Million Women Study Collaborators. 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 362:419–427; doi:10.1016/S0140-6736(03)14065-2.

Million Women Study Collaborators. 2005. Endometrial cancer and hormone-replacement therapy in the Million Women Study. Lancet 365:1543–1551; doi:10.1016/S0140-6736(05)66455-0.

Million Women Study Collaborators. 2007. Ovarian cancer and hormone replacement therapy in the Million Women Study. Lancet 369:1703–1710; doi:10.1016/S0140-6736(07)60534-0.

Moos RK, Angerer J, Dierkes G, Brüning T, Koch HM. 2016. Metabolism and elimination of methyl, *iso*- and *n*-butyl paraben in human urine after single oral dosage. Arch. Toxicol. 90:2699–2709; doi:10.1007/s00204-015-1636-0.

Muir C, Spironello-Vella E, Pisani N, deCatanzaro D. 2001. Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from

male and female mice. Horm. Metab. Res. 33:653–658; doi:10.1055/s-2001-18692.

NIH. 2010. National Center for Biotechnology Information; PubChem BioAssay Database; AID=893. Available: https://pubchem.ncbi.nlm.nih.gov/bioassay/893 [accessed 6 March 2017].

Ozaki H, Sugihara K, Watanabe Y, Ohta S, Kitamura S. 2016. Cytochrome P450-inhibitory activity of parabens and phthalates used in consumer products. J. Toxicol. Sci. 41: 551–560.

Pollock T, deCatanzaro D. 2014. Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses. Reprod. Toxicol. 49:145–154; doi:10.1016/j.reprotox.2014.08.005.

Pollock T, Greville LJ, Tang B, deCatanzaro D. 2016. Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice. Reprod. Toxicol. 65:394–401; doi:10.1016/j.reprotox.2016.09.004.

Pollock T, Mantella L, Reali V, deCatanzaro D. 2017a. Influence of tetrabromobisphenol A, with or without concurrent triclosan, upon bisphenol A and estradiol concentrations in mice. Environ. Health Perspect. In press.

Pollock T, Tang B, deCatanzaro D. 2014. Triclosan exacerbates the presence of ^{14}C-bisphenol A in tissues of female and male mice. Toxicol. Appl. Pharmacol. 278:116–123; doi:10.1016/j.taap.2014.04.017.

Pollock T, Weaver RE, Ghasemi R, deCatanzaro D. 2017b. Butyl paraben and propyl paraben modulate bisphenol A and estradiol concentrations in female and male mice. Toxicol. Appl. Pharmacol. 325:18–24; doi:10.1016/j.taap.2017.04.001.

Pritchett JJ, Kuester RK, Sipes IG. 2002. Metabolism of bisphenol A in primary cultured hepatocytes from mice, rats, and humans. Drug Metab Dispos 30: 1180–1185.

Prusakiewicz JJ, Harville HM, Zhang Y, Ackermann C, Voorman RL. 2007. Parabens inhibit human skin estrogen sulfotransferase activity: Possible link to paraben estrogenic effects. Toxicology 232:248–256; doi:10.1016/j.tox.2007.01.010.

R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available: https://www.r-project.org/.

Rochester JR. 2013. Bisphenol A and human health: A review of the literature. Reprod. Toxicol. 42:132–155; doi:10.1016/j.reprotox.2013.08.008.

Sanders JM, Coulter SJ, Knudsen GA, Dunnick JK, Kissling GE, Birnbaum LS. 2016. Disruption of estrogen homeostasis as a mechanism for uterine toxicity in Wistar Han rats treated with tetrabromobisphenol A. Toxicol. Appl. Pharmacol. 298:31–39; doi:10.1016/j.taap.2016.03.007.

Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, Keri RA. 2016. A review of the carcinogenic potential of bisphenol A. Reprod. Toxicol. 59:167–182; doi:10.1016/j.reprotox.2015.09.006.

Silva MJ, Barr DB, Reidy JA, Kato K, Malek NA, Hodge CC, et al. 2003. Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. Arch. Toxicol. 77:561–567; doi:10.1007/s00204-003-0486-3.

Thorpe JB, Burgess PS, Sadkowski M, deCatanzaro D. 2013. Estrogen-progesterone balance in the context of blastocyst implantation failure induced by predator stress. Psychoneuroendocrinology 38:3048–3056; doi:10.1016/j.psyneuen.2013.09.001.

Thorpe JB, Gould KE, Borman ED, deCatanzaro D. 2014. Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*). Horm. Metab. Res. 46:211–218; doi:10.1055/s-0033-1363958.

U.S. EPA. 2012. Endocrine Disruptor Screening Program: Universe of Chemicals. Available: https://www.epa.gov/sites/production/files/2015-07/documents/edsp_chemical_universe_list_11_12.pdf [accessed 3 May 2017].

Wang L-Q, Falany CN, James MO. 2004. Triclosan as a substrate and inhibitor of 3′-phosphoadenosine 5′-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. Pharmacology 32:1162–1169; doi:10.1124/dmd.104.000273.the.

Wikoff DS, Rager JE, Haws LC, Borghoff SJ. 2016. A high dose mode of action for tetrabromobisphenol A-induced uterine adenocarcinomas in Wistar Han rats: A critical evaluation of key events in an adverse outcome pathway framework. Regul. Toxicol. Pharmacol. 77:143–159; doi:10.1016/j.yrtph.2016.01.018.

Witzmann F, Coughtrie M, Fultz C, Lipscomb J. 1996. Effect of structurally diverse peroxisome proliferators on rat hepatic sulfotransferase. Chem. Biol. Interact. 99:73–84; doi:10.1016/0009-2797(95)03661-X.

Wróbel A, Gregoraszczuk EL. 2013. Effects of single and repeated *in vitro* exposure of three forms of parabens, methyl-, butyl- and propylparabens on the proliferation and estradiol secretion in MCF-7 and MCF-10A cells. Pharmacol. Reports 65:484–493; doi:10.1016/S1734-1140(13)71024-7.

Wu Y, Chitranshi P, Loukotková L, Gamboa da Costa G, Beland FA, Zhang J, et al. 2017. Cytochrome P450-mediated metabolism of triclosan attenuates its cytotoxicity in hepatic cells. Arch. Toxicol. 91:2405–2423; doi:10.1007/s00204-016-1893-6.

Yalcin EB, Kulkarni SR, Slitt AL, King R. 2016. Bisphenol A sulfonation is impaired in metabolic and liver disease. Toxicol. Appl. Pharmacol. 292:75–84; doi:10.1016/j.taap.2015.12.009.

Zalko D, Soto AM, Dolo L, Dorio C, Rathahao E, Debrauwer L, et al. 2003. Biotransformations of bisphenol A in a mammalian model: Answers and new questions raised by low-dose metabolic fate studies in pregnant CD1 mice. Environ. Health Perspect. 111:309–319; doi:10.1289/ehp.5603.

Ziv-Gal A, Flaws JA. 2016. Evidence for bisphenol A-induced female infertility: A review (2007–2016). Fertil. Steril. 106:827–856; doi:10.1016/j.fertnstert.2016.06.027.

Zota AR, Calafat AM, Woodruff TJ. 2014. Temporal trends in phthalate exposures: Findings from the national health and nutrition examination survey, 2001-2010. Environ. Health Perspect. 122:235–241; doi:10.1289/ehp.1306681.

Chapter 8

General Discussion

Contributions of this work to the field

The findings presented in this thesis contribute several key advancements to the field of toxicology. For the first time, I demonstrated that very low doses of dietary BPA, as low as 1/100th of the oral reference dose, can bind to ER in the uterus and that BPA accumulates there with repeated exposures. I also developed a novel protocol that efficiently examined interactions among EDCs in bodily fluids and tissues of rodents. Whereas *in vitro* or *in silico* work can establish the capacity of chemicals to bind a purified receptor or inhibit metabolic enzyme activity towards a specific substrate, *in vivo* work is required to demonstrate that chemicals actually possess biological activity.

I have demonstrated that diverse chemicals can elevate concentrations of BPA and E2 *in vivo*. These effects are most pronounced in the reproductive organs and blood serum, and can be induced by administration of a single chemical or multiple chemicals given concurrently at lower doses. Thus, exposure to numerous chemicals could potentially have adverse effects at doses that are much closer to human exposure than previously assumed. Given established endocrine-disrupting effects of BPA, it is especially concerning that these effects could be amplified by the presence of other chemicals. These findings also show that chemicals can produce strong estrogenic effects by indirectly disrupting estrogen and xenoestrogen metabolism. This alternative mechanism is especially important for chemicals that have low affinity for ER, such as triclosan and TBBPA, which might otherwise be overlooked when assessing endocrine-disrupting effects.

Summary of results

In **Chapter 2**, I showed that BPA distributes throughout the bodies of female rodents. Dietary administration of 50 μg/kg ^{14}C-BPA to cycling female Long-Evans rats distributed into blood serum and throughout peripheral tissues, including the heart, lungs, muscle, adipose, uterus, ovaries, liver, and kidneys. Except for the liver and kidneys, which are involved in metabolism and excretion, levels of radioactivity were greatest in the uterus. Consistent with BPA binding to ER in the uterus, radioactivity was significantly reduced by pre-treatment with either E2 or the ER antagonist ICI 182,780. Radioactivity was also detected in some brain areas, including the olfactory bulb, cerebellum, striatum, hippocampus, and hypothalamus; however, concentrations were 2–3 orders of magnitude lower than those measured in peripheral tissues. Given high variance for measures in cycling females, especially in reproductive organs, I replicated the above experiment in recently inseminated females on gestation day 3. Levels of radioactivity were once again greatest in the uterus and significantly diminished by pre-treatment with E2 or ICI 182,780. One notable difference from the cycling females was that levels of radioactivity in inseminated females were higher in neural tissues.

Measures of radioactivity represent total BPA, including unconjugated and conjugated forms. Given that only unconjugated BPA can bind to ER, I quantified concentrations of unconjugated and total BPA in the uterus using gas chromatography-mass spectrometry (GC-MS). Five cycling female rats were each administered a dose of 50 μg/kg unlabelled BPA. On average, 71.9% of BPA (5.77 of 8.07 ng BPA/g uterine tissue) was unconjugated. Notably, there was a difference in mean concentrations of total

BPA in females given 50 μg/kg ^{14}C-BPA measured via liquid scintillation counting (1.97 ng/g) and those given 50 μg/kg unlabelled BPA measured via GC-MS (8.07 ng/g). Contamination of BPA from equipment and solvents in GC-MS procedures was ruled out via measurement of blank samples. Thus, the only explanation for this striking difference is due to BPA exposure from external sources, such as food, water, bedding, and dust.

Given low concentrations of BPA in neural tissues, we opted to use mice for cost-efficiency. Dietary administration of 50 μg/kg ^{14}C-BPA to cycling female CF-1 mice distributed into blood serum and throughout peripheral tissues, including the heart, lungs, muscle, adipose, uterus, ovaries, liver, and kidneys. Concentrations of BPA were once again greatest in the uterus and approximately 2–3 orders of magnitude lower in brain areas such as the olfactory bulbs, cerebellum, cortex, and hypothalamus. BPA accumulated in the uterus, ovaries, liver, and kidney following 7 or 28 repeated daily doses. Distribution of BPA was dose-dependent, with dietary administration of 5 μg/kg ^{14}C-BPA producing BPA concentrations that were approximately 3–7 times lower than those observed following exposure to 50 μg/kg. Concentrations of BPA were still highest in the uterus relative to other peripheral tissues, whereas BPA was not present in any neural tissue samples. Only the uterus, liver, and kidney showed detectable BPA concentrations following dietary administration of 0.5 μg/kg ^{14}C-BPA.

In **Chapter 3**, I provided evidence that triclosan can modulate concentrations of BPA in mice. These studies were inspired by previous research showing that BPA and triclosan could disrupt blastocyst implantation when given concurrently at lower doses than those required when each chemical was given alone (Crawford and deCatanzaro

2012). Here, animals were injected with a single dose of 0.2–18 mg triclosan followed by dietary administration 50 μg/kg ^{14}C-BPA. Elevated concentrations of BPA were observed in blood serum and peripheral tissues, but not neural tissues, of female and male mice. Similar elevations were observed in blood serum and ovaries of animals treated with 2–6 mg triclosan followed by 5 μg/kg ^{14}C-BPA.

In research presented in **Chapter 4**, I demonstrated that triclosan can elevate concentrations of exogenous ^{3}H-E2 and natural urinary E2 in cycling and recently inseminated female mice. Assessing the impact of triclosan on exogenous ^{3}H-E2 is relevant to human estrogen supplementation, as in hormonal contraceptives and hormone-replacement therapy. Daily injections of 2 mg triclosan for 7 days elevated ^{3}H-E2 concentrations in the heart, lungs, muscle, uterus, ovaries, and blood serum of cycling females. A single injection of 1 or 2 mg triclosan elevated ^{3}H-E2 concentrations in the uterus of cycling females or increased E2 in urine at 6–10 h after injection. Similarly, a single injection of 2 mg triclosan elevated uterine ^{3}H-E2 in recently inseminated females on gestation day 3 or increased E2 in urine at 2–12 h after injection.

In data presented in **Chapter 5**, I showed that BP and PP can alter distribution of dietary ^{14}C-BPA, and that BP can modulate natural urinary E2 concentrations in mice. A single injection of 9 mg BP was required to elevate BPA concentrations in the lungs, ovaries, kidneys, and blood serum of diestrous females, whereas 1–9 mg BP was sufficient to increase BPA concentrations in the uterus. A single dose of 9 mg BP elevated concentrations of BPA in the testes of males, whereas 3–9 mg BP increased BPA concentrations in the epididymides and blood serum. 3 mg BP also increased natural

E2 in urine at 6–10 h after injection in diestrous females and at 8 h after injection in males. Relative to BP, PP was a much weaker chemical, consistent with *in vitro* potency of metabolic enzyme inhibition (Ozaki et al. 2016; Prusakiewicz et al. 2007). A single injection of 9 mg PP elevated BPA concentrations in the uterus of diestrous females, but had no effect on BPA concentrations in males or urinary E2 of either sex.

In **Chapter 6**, I provided evidence that TBBPA can elevate concentrations of dietary ^{14}C-BPA and natural E2 in mice. A single dose of 3–27 mg TBBPA increased BPA concentrations in the heart, lung, muscle, uterus, ovaries, kidneys, and blood serum of diestrous females. A single dose of 1–27 mg TBBPA elevated BPA concentrations in the heart, lung, testes, epididymides, seminal vesicles, preputial glands, kidneys, and blood serum of males. Urinary E2 concentrations were increased by 1 mg TBBPA at 8–12 h after injection in diestrous females and at 2–10 h after injection in males. In **Chapter 6**, I provided evidence that TBBPA and triclosan have additive effects in their capacity to modulate concentrations of dietary ^{14}C-BPA. Whereas a single dose of 0.33 mg of either TBBPA or triclosan produced no effects, concurrent exposure to both 0.33 mg TBBPA and 0.33 mg triclosan elevated BPA concentrations in the uterus, ovaries, and blood serum of diestrous females, as well as the testes, epididymides, and blood serum of males.

In **Chapter 7**, I demonstrated that lower doses of a mixture of 5 EDCs, including triclosan, BP, PP, TBBPA, and DEHP, could disrupt concentrations of dietary ^{14}C-BPA in tissues and E2 in urine of diestrous female, but not male, mice. A single dose of 0.1 mg TBBPA elevated BPA concentrations in the uterus, whereas 0.5 mg TBBPA or 0.5 mg

triclosan produced similar effects in the uterus, ovaries, and blood serum. BP, PP, and DEHP had no such effects at 0.1 or 0.5 mg alone. However, a single dose of the 0.5 mg mixture containing 0.1 mg of all 5 chemicals elevated BPA concentrations in the lungs, muscle, uterus, ovaries, kidneys, and blood serum. The 0.5 mg mixture also increased natural E2 in urine at 8 h after injection.

Differences between sexes in EDC modulation of BPA and E2 concentrations

Table 1 shows the lowest dose of each chemical, administered alone or as a mixture, that was sufficient to elevate ^{14}C-BPA concentrations in tissues and blood serum of female and male mice. This table includes the impact of DEHP on dietary ^{14}C-BPA that was assessed in another study conducted in this laboratory (Borman et al. 2017). Table 2 shows the fold increase in ^{14}C-BPA concentrations in the uterus, epididymides, and blood serum of mice given 1 mg of each chemical relative to vehicle controls. Only the blood serum, uterus, and epididymides were selected for inclusion in this table, as all four chemicals (triclosan, BP, PP, and TBBPA) produced significant effects there. Data for DEHP are not included in this table, as 3 mg was the lowest dose studied (Borman et al. 2017). As evidenced by data in Tables 1 and 2, EDCs have varying impacts in their capacity to modulate BPA concentrations *in vivo*.

Table 1 Lowest dose (in mg), administered via a single subcutaneous injection, that significantly elevated concentrations of dietary ^{14}C-BPA in tissues and blood serum of female and male mice. Blank entries indicate the highest dose assessed was ineffective.

Females	Triclosan	BP	PP	TBBPA	DEHP[a]	Triclosan + TBBPA[b]	Mixture[b,c]
Heart	18			3			
Lungs	0.6	9		3			0.1
Muscle	18			9	18		0.1
Adipose							
Uterus	0.5	1	9	0.1	18	0.33	0.1
Ovaries	0.5	9		0.5	18	0.33	0.1
Liver							
Kidneys		9		3			
Blood Serum	0.5	9		0.5	18	0.33	0.1

Males	Triclosan	BP	PP	TBBPA	DEHP[a]	Triclosan + TBBPA[b]	Mixture[b,c]
Heart				1			
Lungs				9			
Muscle							
Adipose							
Testes		9		3		0.33	
Epididymides	2	3		1	18	0.33	
Seminal Vesicles				1			
Preputial Glands	NA[d]			9			
Liver							
Kidneys				1			
Blood Serum	0.2	3		1	9	0.33	

[a]Data from Borman et al. (2017).
[b]Data represents the dose of each chemical administered concurrently.
[c]Mixture contains triclosan, BP, PP, TBBPA, and DEHP in equal proportions.
[d]Tissue was not assessed (NA).

Table 2 Fold increase in ^{14}C-BPA concentrations in the uterus, epididymides, and blood serum of mice administered 1 mg of each chemical relative to vehicle controls. All animals were fed a peanut butter dietary supplement containing 50 μg/kg ^{14}C-BPA.

Females	**Triclosan**	**BP**	**PP**	**TBBPA**
Uterus	3.25	2.35	1.32	3.08
Blood Serum	2.33	1.67	1.34	2.19
Males				
Epididymides	2.65[a]	1.64	1.08	2.88
Blood Serum	2.38[a]	1.63	1.13	2.43

[a]Data corresponds to 2 mg triclosan, as 1 mg triclosan was not assessed in males.

Females were affected to a greater extent, both in terms of the variety of tissues impacted and the doses required, than were males. This was especially evident in peripheral tissues shared by both sexes, such as the heart, lungs, muscle, and kidneys. Whereas effects of EDCs on BPA concentrations were observed for triclosan, BP, TBBPA, DEHP, and the mixture of five chemicals in at least some of these tissues in females, only TBBPA produced effects in these tissues in males. Except for the mixture of five chemicals in males, BPA concentrations were consistently elevated by EDCs in blood serum and reproductive organs of both sexes. As evidenced in Table 2, exposure to 1–2 mg triclosan, 1 mg BP, 1 mg PP, or 1 mg TBBPA affected blood serum of males and females to a similar extent. In reproductive organs, however, females required lower doses of most chemicals to achieve statistical significance relative to vehicle controls. Elevations in ^{14}C-BPA concentrations in the uterus were of greater magnitude than those in the epididymides. Comparing data between males and females given the same dose of triclosan, BP, PP, or TBBPA may not be meaningful, as doses were given *per animal* rather than *per kg body weight*, as done for ^{14}C-BPA. When doses were calculated based on body weight, females received a higher dose than did males (see Tables 3.1, 5.1, 6.1, and 7.1). For example, 1 mg TBBPA corresponded to an average of 32.7 mg/kg in females but only 24.6 mg/kg in males. In addition to dose discrepancies, differences in BPA concentrations between sexes may also be explained by receptor and metabolic variations. The uterus and ovaries of females and epididymides of males highly express ERα, whereas only the reproductive organs of females highly express ERβ (Kuiper et al. 1997). Metabolism of BPA and other EDCs is impacted by sex- and tissue-specific

expression of numerous UGT isoforms (Buckley and Klaassen 2007); males show faster metabolic rates for certain chemicals, such as TBBPA (Zalko et al. 2006).

Interestingly, no statistically significant effects were observed in the liver or adipose of either sex. Given that the liver is highly involved in metabolism of toxicants, competition among EDCs and BPA there should permit some of the ingested BPA to avoid conjugation (Sieli et al. 2011; Wang et al. 2004). Unconjugated BPA may deposit in the liver to some extent, but can also enter circulation and distribute throughout tissues of rodents (Kim et al. 2004; **Chapter 2**). Small lipophilic molecules such as BPA are traditionally thought to deposit and accumulate in adipose. This was not the case for acute studies assessing the impacts of EDCs on BPA concentrations (**Chapters 3–7**) nor was it the case for daily BPA exposures up to 28 days (**Chapter 2**). A study in humans detected BPA in adipose (Wang et al. 2015); however, samples were obtained from patients undergoing liposuction surgery that may have higher exposure to BPA than the general population (Wang et al. 2012).

Similar to BPA concentrations, EDCs modulate natural E2 concentrations in urine of females to a greater extent than that of males. A single dose of 1 mg TBBPA or 3 mg BP elevated urinary E2 in both female and male mice; however, the magnitude of elevations was greater in females. Furthermore, the mixture of five chemicals only increased E2 concentrations in urine of females. Direct comparisons between sexes cannot be made for other chemicals, as impacts on E2 were not assessed for triclosan in males or DEHP in either sex, and 3 mg PP showed no significant effects in either sex. The latency of EDC actions on urinary E2 occurs up to 12 h after injection. As such,

these findings could be attributed to disruption of estrogen metabolism and/or increased E2 biosynthesis. Higher magnitude of effects in females could be attributed to greater aromatase expression in the ovaries than in the testes (Golovine 2003). Aromatase expression can also be increased in response to certain EDCs, such as TBBPA (Honkisz and Wójtowicz 2015), leading to increased E2 output.

Implications for regulatory decision-making

The oral reference dose for BPA is 50 μg/kg/day and was last revised by the U.S. EPA in 1988 (U.S. EPA 1988). The reference dose for BPA was derived from the lowest observed adverse effect level (LOAEL) of 50 mg/kg/day in a two-year study (NTP 1982). In this study, rats fed diets containing 50 or 100 mg BPA/kg bodyweight/day showed reduced body weights relative to controls (NTP 1982). Doses below 50 mg/kg/day were not assessed. The LOAEL was divided by an uncertainty factor of 1000, which was meant to account for uncertainty associated with chronic exposures, sensitive populations, and extrapolation from animal doses to humans (U.S. EPA 1988). Health Canada established a provisional tolerable daily intake (TDI) of 25 μg/kg/day for BPA in 1996, which was later maintained in 2008 on the basis that it still provided a sufficient margin of safety (Health Canada 2008).

Effects of BPA at doses lower than the oral reference dose prompted some researchers to recommend updated risk assessments to reflect these more recent findings (Vandenberg et al. 2012; vom Saal and Hughes 2005; vom Saal and Welshons 2006; Welshons et al. 2003, 2006). While incorporating all available data is important for

assessing risk, most of these studies still only addressed adverse health outcomes following exposure to a single chemical. The findings of studies conducted as part of this thesis demonstrate that toxicokinetics of one EDC can be modulated by co-exposure to other EDCs. Effects of EDCs may be additive or synergistic, producing adverse health outcomes at lower doses when co-exposed. Indeed, concurrent exposure to triclosan and BPA can block intrauterine implantation of fertilized ova and disrupt pregnancy (Crawford and deCatanzaro 2012), while concurrent exposure to BP and DEHP can disrupt ovarian steroidogenesis, leading to attenuated E2 output (Guerra et al. 2016). It is insufficient to solely consider studies assessing effects of a single chemical for the purposes of assessing risk. Studies investigating effects of exposure to multiple toxicants should be considered when conducting quantitative risk assessments.

Future directions

The experiments reported in this thesis investigated the unidirectional capacity with which certain EDCs could modulate concentrations of dietary ^{14}C-BPA. Measurement of radioactivity via liquid scintillation counting permitted rapid, cost-effective quantification of BPA in blood serum and tissues of mice. Other radiolabeled toxicants are not commercially available and must be custom synthesized at a high cost for low quantities. Other toxicants could also be measured via GC-MS or similar procedures at a high cost per sample, as done in **Chapter 2** for unlabeled BPA. One limitation of this thesis is that the capacity of chemical mixtures to modulate the toxicokinetics of other constituents of the mixture was not assessed. These data would

have been particularly interesting for experiments conducted in **Chapter 7**, when triclosan, BP, PP, TBBPA, and DEHP were administered concurrently. Another limitation of this thesis is that these experiments only examined the capacity of EDCs to modulate concentrations of BPA at one time-point and via one route of exposure. Future studies could more broadly investigate the potential for EDCs to modulate the toxicokinetics of other EDCs, at various time-points and via other routes of exposure.

Given that humans are routinely exposed to many potential EDCs (U.S. EPA 2017b), more toxicological research should focus on assessing effects of exposure to chemical mixtures. Toxicological studies examining effects of a single chemical are particularly useful for determining each chemical's mechanism of action. However, studies of multiple EDCs are necessary to determine potential disruption of endocrine functions. Given that there are over 10,000 unique chemicals included in the EDSP (U.S. EPA 2012a, 2012b), assembling chemical mixtures to be studied may be challenging.

One approach for selecting constituents of chemical mixtures could be to use high-throughput *in vitro* or *in silico* data to determine which EDCs are the most potent. These data would permit researchers and risk assessors to prioritize certain chemicals for evaluation in costly and time-consuming *in vivo* studies. One example of such data is the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP), which used computational modeling to evaluate 32,464 chemicals for ER-related activity (Mansouri et al. 2016; U.S. EPA 2017a). Of these chemicals, 4,001 were identified as high priority actives, some of which might be candidates for further study (Mansouri et al. 2016).

Another approach for selecting constituents of chemical mixtures could be to use NHANES data to determine which EDCs are reliably detected, perhaps at high concentrations, in the same individuals. Although not all potential EDCs are assessed in NHANES, the 2011–2012 project quantified a variety of chemicals, including environmental phenols (BPA, triclosan, and benzophenone-3), parabens (methyl, ethyl, propyl, and butyl), phthalates and phthalate metabolites, pesticides (dichlorophenol), and polyaromatic hydrocarbons (phenanthrene, naphthalene, fluorene, and pyrene). Many of these chemicals are included in the EDSP universe of chemicals (U.S. EPA 2012a). Studies of chemical mixtures that best resemble human exposures would likely be a priority for risk assessors and regulatory agencies.

Conclusion

The data presented in this thesis provide novel insight into interactions among EDCs, as well as their capacity to disrupt natural estrogen homeostasis. Persistently elevated estrogenic activity can impede sexual development, perturb fertility, and promote tumour growth. Thus, continued investigation into interactions among toxicants, especially the adverse health outcomes of exposure to estrogenic substances, is needed. Consideration of alternative mechanisms of estrogenic actions, such as impacts on metabolic enzyme activity, may facilitate understanding of the endocrine-disrupting effects of certain chemicals. Cumulative effects may redefine how we quantify and manage risks of toxicants. It is critical that studies of multiple substances be considered when assessing risks to humans and wildlife and determining regulatory exposure limits.

General References (Chapters 1 and 8)

Abbas S, Greige-Gerges H, Karam N, Piet M-H, Netter P, Magdalou J. 2010. Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man. Drug Metab. Pharmacokinet. 25:568–577; doi:10.2133/dmpk.DMPK-10-RG-013.

Abdallah MA-E, Harrad S. 2011. Tetrabromobisphenol-A, hexabromocyclododecane and its degradation products in UK human milk: Relationship to external exposure. Environ. Int. 37:443–448; doi:10.1016/j.envint.2010.11.008.

Al-Hiyasat AS, Darmani H, Elbetieha AM. 2004. Leached components from dental composites and their effects on fertility of female mice. Eur. J. Oral Sci. 112:267–272; doi:10.1111/j.1600-0722.2004.00136.x.

Allmyr M, Adolfsson-Erici M, McLachlan MS, Sandborgh-Englund G. 2006. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. Sci. Total Environ. 372:87–93; doi:10.1016/j.scitotenv.2006.08.007.

Allmyr M, Harden F, Toms LML, Mueller JF, McLachlan MS, Adolfsson-Erici M, et al. 2008. The influence of age and gender on triclosan concentrations in Australian human blood serum. Sci. Total Environ. 393:162–167; doi:10.1016/j.scitotenv.2007.12.006.

Ames B. 2013. Tetrabrominated bisphenol A inhibition studies on six cytochrome P450 enzymes in human liver microsomes. California State University (dissertation).

Andersen FA. 2008. Final amended report on the safety assessment of methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, and benzylparaben as used in cosmetic products. Int. J. Toxicol. 27:1–82; doi:10.1080/10915810500257113.

Anderson DC. 1974. Sex-hormone-binding globulin. Clin. Endocrinol. 3:69–96.

Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. 2006. Position statement: Criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: An androgen excess society guideline. J. Clin. Endocrinol. Metab. 91:4237–4245; doi:10.1210/jc.2006-0178.

Barton M, Filardo EJ, Lolait SJ, Thomas P, Maggiolini M, Prossnitz ER. 2017. Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. J. Steroid Biochem. Mol. Biol. 1–12; doi:10.1016/j.jsbmb.2017.03.021.

Berger RG, Foster WG, deCatanzaro D. 2010. Bisphenol-A exposure during the period of blastocyst implantation alters uterine morphology and perturbs measures of estrogen and progesterone receptor expression in mice. Reprod. Toxicol. 30:393–400; doi:10.1016/j.reprotox.2010.06.006.

Berger RG, Shaw J, deCatanzaro D. 2008. Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol. Reprod. Toxicol. 26:94–99; doi:10.1016/j.reprotox.2008.06.007.

Bhasin S, Cunningham GR, Hayes FJ, Matsumoto AM, Snyder PJ, Swerdloff RS, et al. 2010. Testosterone therapy in men with androgen deficiency syndromes: An endocrine society clinical practice guideline. J. Clin. Endocrinol. Metab. 95:2536–2559; doi:10.1210/jc.2009-2354.

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, et al. 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. Toxicol. Sci. 54:138–153; doi:10.1093/toxsci/54.1.138.

Borman ED, Vecchi N, Pollock T, deCatanzaro D. 2017. Diethylhexyl phthalate magnifies deposition of ^{14}C-bisphenol A in reproductive tissues of mice. J. Appl. Toxicol. In press; doi:10.1002/jat.3484.

Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. 1995. Xenoestrogens released from lacquer coatings in food cans. Environ. Health Perspect. 103:608–612; doi:10.1289/ehp.95103608.

Buckley DB, Klaassen CD. 2007. Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. Drug Metab. Dispos. 35:121–127; doi:10.1124/dmd.106.012070.

Byford JR, Shaw LE, Drew MGB, Pope GS, Sauer MJ, Darbre PD. 2002. Oestrogenic activity of parabens in MCF7 human breast cancer cells. J. Steroid Biochem. Mol. Biol. 80:49–60; doi:10.1016/S0960-0760(01)00174-1.

Calafat AM, Ye X, Wong LY, Bishop AM, Needham LL. 2010. Urinary concentrations of four parabens in the U.S. Population: NHANES 2005–2006. Environ. Health Perspect. 118:679–685; doi:10.1289/ehp.0901560.

Cariou R, Antignac J-P, Zalko D, Berrebi A, Cravedi J-P, Maume D, et al. 2008. Exposure assessment of French women and their newborns to tetrabromobisphenol-A: Occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum. Chemosphere 73:1036–1041; doi:10.1016/j.chemosphere.2008.07.084.

Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, et al. 2006. Genome-wide analysis of estrogen receptor binding sites. Nat. Genet. 38:1289–1297; doi:10.1038/ng1901.

CDC. 2015. Fourth national report on human exposure to environmental chemicals. Available: https://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Aug2014.pdf [accessed 10 November 2016].

Chen D, Kannan K, Tan H, Zheng Z, Feng YL, Wu Y, et al. 2016. Bisphenol analogues other than BPA: Environmental occurrence, human exposure, and toxicity – A review. Environ. Sci. Technol. 50:5438–5453; doi:10.1021/acs.est.5b05387.

Chumsri S, Howes T, Bao T, Sabnis G, Brodie A. 2011. Aromatase, aromatase inhibitors, and breast cancer. J. Steroid Biochem. Mol. Biol. 125:13–22; doi:10.1016/j.jsbmb.2011.02.001.

Ciocca DR, Fanelli MA. 1997. Estrogen receptors and cell proliferation in breast cancer. Trends Endocrinol. Metab. 8: 313–321.

Colnot T, Kacew S, Dekant W. 2014. Mammalian toxicology and human exposures to the flame retardant 2,2′,6,6′-tetrabromo-4,4′-isopropylidenediphenol (TBBPA): Implications for risk assessment. Arch. Toxicol. 88:553–573; doi:10.1007/s00204-013-1180-8.

Crawford BR, deCatanzaro D. 2012. Disruption of blastocyst implantation by triclosan in mice: Impacts of repeated and acute doses and combination with bisphenol-A. Reprod. Toxicol. 34:607–613; doi:10.1016/j.reprotox.2012.09.008.

Csapo A, Pohanka O, Kaihola H. 1974. Progesterone deficiency and premature labour. Br. Med. J. 1:137–40; doi:10.1136/bmj.1.5899.137.

Darbre PD, Aljarrah A, Miller WR, Coldham NG, Sauer MJ, Pope GS. 2004. Concentrations of parabens in human breast tumours. J. Appl. Toxicol. 24:5–13; doi:10.1002/jat.958.

deCatanzaro D, Baptista MAS, Spironello-Vella E. 2001. Administration of minute quantities of 17β-estradiol on the nasal area terminates early pregnancy in inseminated female mice. Pharmacol. Biochem. Behav. 69:503–509; doi:10.1016/S0091-3057(01)00554-8.

Dong S, Terasaka S, Kiyama R. 2011. Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. Environ. Pollut. 159:212–218; doi:10.1016/j.envpol.2010.09.004.

Dorosh A, Děd L, Elzeinová F, Pěknicová J. 2011. Assessing oestrogenic effects of brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on MCF-7 cells. Folia Biol 57: 35–39.

Dumas I, Diorio C. 2011. Estrogen pathway polymorphisms and mammographic density. Anticancer Res. 31: 4369–4386.

EFSA. 2015. Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA J. 13:3978; doi:10.2903/j.efsa.2015.3978.

Ehrlich S, Calafat AM, Humblet O, Smith T, Hauser R. 2014. Handling of thermal receipts as a source of exposure to bisphenol A. J. Am. Med. Assoc. 311:859–860; doi:10.1001/jama.2013.283735.

Etzel TM, Calafat AM, Ye X, Chen A, Lanphear BP, Savitz DA, et al. 2017. Urinary triclosan concentrations during pregnancy and birth outcomes. Environ. Res. 156:505–511; doi:10.1016/j.envres.2017.04.015.

Fang J-L, Stingley RL, Beland FA, Harrouk W, Lumpkins DL, Howard P. 2010. Occurrence, efficacy, metabolism, and toxicity of triclosan. J. Environ. Sci. Heal. Part C 28:147–171; doi:10.1080/10590501.2010.504978.

Filardo EJ, Thomas P. 2005. GPR30: A seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol. Metab. 16:362–367; doi:10.1016/j.tem.2005.08.005.

Fossum GT, Davidson A, Paulson RJ. 1989. Ovarian hyperstimulation inhibits embryo implantation in the mouse. J. Vitr. Fertil. Embryo Transf. 6:7–10; doi:10.1007/BF01134574.

Frederiksen H, Jørgensen N, Andersson A-M. 2011. Parabens in urine, serum and seminal plasma from healthy Danish men determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS). J. Expo. Sci. Environ. Epidemiol. 21:262–271; doi:10.1038/jes.2010.6.

Fujii Y, Nishimura E, Kato Y, Harada KH, Koizumi A, Haraguchi K. 2014. Dietary exposure to phenolic and methoxylated organohalogen contaminants in relation to their concentrations in breast milk and serum in Japan. Environ. Int. 63:19–25; doi:10.1016/j.envint.2013.10.016.

Garcia T, Schreiber E, Kumar V, Prasad R, Sirvent JJ, Domingo JL, et al. 2017. Effects on the reproductive system of young male rats of subcutaneous exposure to n-butylparaben. Food Chem. Toxicol. 106:47–57; doi:10.1016/j.fct.2017.05.031.

Gee RH, Charles A, Taylor N, Darbre PD. 2008. Oestrogenic and androgenic activity of triclosan in breast cancer cells. J. Appl. Toxicol. 28:78–91; doi:10.1002/jat.1316.

Geens T, Neels H, Covaci A. 2012. Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. Chemosphere 87:796–802; doi:10.1016/j.chemosphere.2012.01.002.

Geens T, Roosens L, Neels H, Covaci A. 2009. Assessment of human exposure to bisphenol-A, triclosan and tetrabromobisphenol-A through indoor dust intake in Belgium. Chemosphere 76:755–760; doi:10.1016/j.chemosphere.2009.05.024.

Gidley-Baird AA, O'Neill C, Sinosich MJ, Porter RN, Pike IL, Saunders DM. 1986. Failure of implantation in human in vitro fertilization and embryo transfer patients: the effects of altered progesterone/estrogen ratios in humans and mice. Fertil. Steril. 45:69–74; doi:10.1016/S0015-0282(16)49099-0.

Golovine K, Schwerin M, Vanselow J. 2003. Three different promoters control expression of the aromatase cytochrome P450 gene (*Cyp19*) in mouse gonads and brain. Biol. Reprod. 68:978–984; doi:10.1095/biolreprod.102.008037.

Gomez E, Pillon A, Fenet H, Rosain D, Duchesne MJ, Nicolas JC, et al. 2005. Estrogenic activity of cosmetic components in reporter cell lines: Parabens, UV screens, and musks. J. Toxicol. Environ. Health. A 68:239–251; doi:10.1080/15287390590895054.

Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. 2015. EDC-2: The Endocrine Society's second scientific statement on endocrine-disrupting chemicals. Endocr. Rev. 36:593–602; doi:10.1210/er.2015-1093.

Gosavi RA, Knudsen GA, Birnbaum LS, Pedersen LC. 2013. Mimicking of estradiol binding by flame retardants and their metabolites: A crystallographic analysis. Environ. Health Perspect. 121:1194–1199; doi:10.1289/ehp.1306902.

Guerra MT, Furlong HC, Kempinas WG, Foster WG. 2016. Effects of *in vitro* exposure to butylparaben and di-(2 ethylhexyl) phthalate, alone or in combination, on ovarian function. J. Appl. Toxicol. 36:1235–1245; doi:10.1002/jat.3335.

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MHA, Andersson PL, et al. 2006. *In vitro* profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol. Sci. 92:157–173; doi:10.1093/toxsci/kfj187.

Han C, Lim YH, Hong YC. 2016. Ten-year trends in urinary concentrations of triclosan and benzophenone-3 in the general U.S. population from 2003 to 2012. Environ. Pollut. 208:803–810; doi:10.1016/j.envpol.2015.11.002.

Hanioka N, Isobe T, Kinashi Y, Tanaka-Kagawa T, Jinno H. 2016. Hepatic and intestinal glucuronidation of mono(2-ethylhexyl) phthalate, an active metabolite of di(2-ethylhexyl) phthalate, in humans, dogs, rats, and mice: An *in vitro* analysis using microsomal fractions. Arch. Toxicol. 90:1651–1657; doi:10.1007/s00204-015-1619-1.

Hanioka N, Takahara Y, Takahara Y, Tanaka-Kagawa T, Jinno H, Narimatsu S. 2012. Hydrolysis of di-*n*-butyl phthalate, butylbenzyl phthalate and di(2-ethylhexyl) phthalate in human liver microsomes. Chemosphere 89:1112–1117; doi:10.1016/j.chemosphere.2012.05.095.

Hanukoglu I. 1992. Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. Steroid Biochem. Molec. Biol 43:779–804; doi:10.1016/0960-0760(92)90307-5.

Harju M, Hamers T, Kamstra JH, Sonneveld E, Boon JP, Tysklind M, et al. 2007. Quantitative structure–activity relationship modeling on *in vitro* endocrine effects and metabolic stability involving 26 selected brominated flame retardants. Environ. Toxicol. Chem. 26:816–826; doi:10.1897/06-308R.1.

Health Canada. 2008. Health risk assessment of bisphenol A from food packaging applications. Available: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/packaging-materials/bisphenol/health-risk-assessment-bisphenol-food-packaging-applications.html [accessed 18 April 2017].

Heath RJ, Rubin JR, Holland DR, Zhang E, Snow ME, Rock CO. 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. J. Biol. Chem. 274:11110–11114; doi:10.1074/jbc.274.16.11110.

Ho KL, Yuen KK, Yau MS, Murphy MB, Wan Y, Fong BMW, et al. 2017. Glucuronide and sulfate conjugates of tetrabromobisphenol A (TBBPA): Chemical synthesis and correlation between their urinary levels and plasma TBBPA content in voluntary human donors. Environ. Int. 98:46–53; doi:10.1016/j.envint.2016.09.018.

Honkisz E, Wójtowicz AK. 2015. Modulation of estradiol synthesis and aromatase activity in human choriocarcinoma JEG-3 cells exposed to tetrabromobisphenol A. Toxicol. In Vitro 29:44–50; doi:10.1016/j.tiv.2014.09.003.

Hua R, Zhou Y, Wu B, Huang Z, Zhu Y, Song Y, et al. 2017. Urinary triclosan concentrations and early outcomes of *in vitro* fertilization-embryo transfer. Reproduction 153:319–325; doi:10.1530/REP-16-0501.

James MO, Ambadapadi S, Falany C. 2015. Triclosan inhibits the activity of expressed human sulfotransferases (SULTs) towards their diagnostic substrates. FASEB J. 29: 622 (Abstract).

James MO, Li W, Summerlot DP, Rowland-Faux L, Wood CE. 2010. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ. Int. 36:942–949; doi:10.1016/j.envint.2009.02.004.

Jiménez-Díaz I, Vela-Soria F, Zafra-Gómez A, Navalón A, Ballesteros O, Navea N, et al. 2011. A new liquid chromatography-tandem mass spectrometry method for determination of parabens in human placental tissue samples. Talanta 84:702–709; doi:10.1016/j.talanta.2011.01.075.

Johnson-Restrepo B, Adams DH, Kannan K. 2008. Tetrabromobisphenol A (TBBPA) and hexabromocyclododecanes (HBCDs) in tissues of humans, dolphins, and sharks from the United States. Chemosphere 70:1935–1944; doi:10.1016/j.chemosphere.2007.10.002.

Jung EM, An BS, Choi KC, Jeung EB. 2012. Potential estrogenic activity of triclosan in the uterus of immature rats and rat pituitary GH3 cells. Toxicol. Lett. 208:142–148; doi:10.1016/j.toxlet.2011.10.017.

Kester MHA, Bulduk S, van Toor H, Tibboel D, Meinl W, Glatt H, et al. 2002. Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J. Clin. Endocrinol. Metab. 87:1142–1150; doi:10.1210/jcem.87.3.8311.

Kim CS, Sapienza PP, Ross IA, Johnson W, Luu HMD, Hutter JC. 2004. Distribution of bisphenol A in the neuroendocrine organs of female rats. Toxicol. Ind. Health 20:41–50; doi:10.1191/0748233704th186oa.

Kim JY, Yi BR, Go RE, Hwang KA, Nam KH, Choi KC. 2014. Methoxychlor and triclosan stimulates ovarian cancer growth by regulating cell cycle- and apoptosis-related genes via an estrogen receptor-dependent pathway. Environ. Toxicol. Pharmacol. 37:1264–1274; doi:10.1016/j.etap.2014.04.013.

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, et al. 2005. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol. Sci. 84:249–259; doi:10.1093/toxsci/kfi074.

Klinge CM. 2001. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 29:2905–2919; doi:10.1093/nar/29.14.2905.

Kota SK, Gayatri K, Jammula S, Kota SK, Krishna SVS, Meher LK, et al. 2013. Endocrinology of parturition. Indian J. Endocrinol. Metab. 17:50–59; doi:10.4103/2230-8210.107841.

Krishnan A V, Stathis P, Permuth SF, Tokes L, Feldman D. 1993. Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279–2286; doi:10.1210/endo.132.6.8504731.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138: 863–870.

Kurebayashi H, Nagatsuka SI, Nemoto H, Noguchi H, Ohno Y. 2005. Disposition of low doses of ^{14}C-bisphenol A in male, female, pregnant, fetal, and neonatal rats. Arch. Toxicol. 79:243–252; doi:10.1007/s00204-004-0628-2.

Lee HK, Kim TS, Kim CY, Kang IH, Kim MG, Jung KK, et al. 2012. Evaluation of *in vitro* screening system for estrogenicity: Comparison of stably transfected human estrogen receptor-α transcriptional activation (OECD TG455) assay and estrogen receptor (ER) binding assay. J. Toxicol. Sci. 37:431–437; doi:10.2131/jts.37.431.

Lemini C, Hernández A, Jaimez R, Franco Y, Avila ME, Castell A. 2004. Morphometric analysis of mice uteri treated with the preservatives methyl, ethyl, propyl, and butylparaben. Toxicol. Ind. Health 20:123–132; doi:10.1191/0748233704th202oa.

Li J, Ma M, Wang Z. 2010. *In vitro* profiling of endocrine disrupting effects of phenols. Toxicol. In Vitro 24:201–207; doi:10.1016/j.tiv.2009.09.008.

Li R, Yu C, Gao R, Liu X, Lu J, Zhao L, et al. 2012. Effects of DEHP on endometrial receptivity and embryo implantation in pregnant mice. J. Hazard. Mater. 241–242:231–240; doi:10.1016/j.jhazmat.2012.09.038.

Liu T, Jia Y, Zhou L, Wang Q, Sun D, Xu J, et al. 2016. Effects of di-(2-ethylhexyl) phthalate on the hypothalamus-uterus in pubertal female rats. Int. J. Environ. Res. Public Health 13:1130; doi:10.3390/ijerph13111130.

Louis GW, Hallinger DR, Stoker TE. 2013. The effect of triclosan on the uterotrophic response to extended doses of ethinyl estradiol in the weanling rat. Reprod. Toxicol. 36:71–77; doi:10.1016/j.reprotox.2012.12.001.

Maltais R, Poirier D. 2011. Steroid sulfatase inhibitors: A review covering the promising 2000-2010 decade. Steroids 76:929–948; doi:10.1016/j.steroids.2011.03.010.

Mansouri K, Abdelaziz A, Rybacka A, Roncaglioni A, Tropsha A, Varnek A, et al. 2016. CERAPP: Collaborative Estrogen Receptor Activity Prediction Project. Environ. Health Perspect. 124:1023–1033; doi:10.1289/ehp.1510267.

Mariana M, Feiteiro J, Verde I, Cairrao E. 2016. The effects of phthalates in the cardiovascular and reproductive systems: A review. Environ. Int. 94:758–776; doi:10.1016/j.envint.2016.07.004.

Matsushima A, Kakuta Y, Teramoto T, Koshiba T, Liu X, Okada H, et al. 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERRγ. J. Biochem. 142:517–524; doi:10.1093/jb/mvm158.

McDonnell DP. 2002. Connections and regulation of the human estrogen receptor. Science. 296:1642–1644; doi:10.1126/science.1071884.

Meerts IA, Letcher RJ, Hoving S, Marsh G, Bergman A, Lemmen JG, et al. 2001. *In vitro* estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. Environ. Health Perspect. 109: 399–407.

Miller D, Wheals BB, Beresford N, Sumpter JP. 2001. Estrogenic activity of phenolic additives determined by an *in vitro* yeast bioassay. Environ. Health Perspect. 109: 133–138.

Miller WL. 1988. Molecular biology of steroid hormone synthesis. Endocr. Rev. 9: 295–318.

Million Women Study Collaborators. 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 362:419–427; doi:10.1016/S0140-6736(03)14065-2.

Million Women Study Collaborators. 2005. Endometrial cancer and hormone-replacement therapy in the Million Women Study. Lancet 365:1543–1551; doi:10.1016/S0140-6736(05)66455-0.

Million Women Study Collaborators. 2007. Ovarian cancer and hormone replacement therapy in the Million Women Study. Lancet 369:1703–1710; doi:10.1016/S0140-6736(07)60534-0.

Molina-Molina JM, Amaya E, Grimaldi M, Sáenz JM, Real M, Fernández MF, et al. 2013. *In vitro* study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. Toxicol. Appl. Pharmacol. 272:127–136; doi:10.1016/j.taap.2013.05.015.

Nakao T, Akiyama E, Kakutani H, Mizuno A, Aozasa O, Akai Y, et al. 2015. Levels of tetrabromobisphenol A, tribromobisphenol A, dibromobisphenol A, monobromobisphenol A, and bisphenol A in Japanese breast milk. Chem. Res. Toxicol. 28:722–728; doi:10.1021/tx500495j.

Nelson RJ, Kriegsfeld LJ. 2017. *An introduction to behavioral endocrinology*. 5th ed. Sinauer Associates Inc., Sunderland, Massachusetts.

NIH. 2010. National Center for Biotechnology Information; PubChem BioAssay Database; AID=893. Available: https://pubchem.ncbi.nlm.nih.gov/bioassay/893 [accessed 6 March 2017].

NTP. 1982. Technical report on the carcinogenesis bioassay of bisphenol A (CAS No. 80-05-7) in F344 rats and B6C3F1 mice (feed study). NTP-80-35. NIH Publ. No. 82-1771.

NTP. 2014. Technical report on the toxicology studies of tetrabromobisphenol A (CAS NO. 79-94-7) in F344/NTac rats and B6C3F1/N mice and toxicology and carcinogenesis studies of tetrabromobisphenol A in Wistar Han [Crl:WI(Han)] rats and B6C3F1/N mice. Available: https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr587_508.pdf [accessed 8 March 2017].

Oishi S. 2002a. Effects of butyl paraben on the male reproductive system in mice. Arch. Toxicol. 76:423–429; doi:10.1007/s00204-002-0360-8.

Oishi S. 2002b. Effects of propyl paraben on the male reproductive system. Food Chem. Toxicol. 40:1807–1813; doi:10.1016/S0278-6915(02)00204-1.

Okada H, Tokunaga T, Liu X, Takayanagi S, Matsushima A, Shimohigashi Y. 2008. Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-γ. Environ. Health Perspect. 116:32–38; doi:10.1289/ehp.10587.

Olsen CM, Meussen-Elholm ETM, Samuelsen M, Holme JA, Hongslo JK. 2003. Effects of the environmental oestrogens bisphenol A, tetrachlorobisphenol A, tetrabromobisphenol A, 4-hydroxybiphenyl and 4,4’-dihydroxybiphenyl on oestrogen receptor binding, cell proliferation and regulation of oestrogen sensitive proteins. Pharmacol. Toxicol. 92: 180–188.

Ozaki H, Sugihara K, Watanabe Y, Ohta S, Kitamura S. 2016. Cytochrome P450-inhibitory activity of parabens and phthalates used in consumer products. J. Toxicol. Sci. 41: 551–560.

Payne AH, Hales DB. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25:947–970; doi:10.1210/er.2003-0030.

Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. 2008. Estrogen signaling through the transmembrane G protein–coupled receptor GPR30. Annu. Rev. Physiol. 70:165–190; doi:10.1146/annurev.physiol.70.113006.100518.

Prusakiewicz JJ, Harville HM, Zhang Y, Ackermann C, Voorman RL. 2007. Parabens inhibit human skin estrogen sulfotransferase activity: Possible link to paraben estrogenic effects. Toxicology 232:248–256; doi:10.1016/j.tox.2007.01.010.

Reed MJ, Purohit A, Woo LWL, Newman SP, Potter BVL. 2005. Steroid sulfatase: Molecular biology, regulation, and inhibition. Endocr. Rev. 26:171–202; doi:10.1210/er.2004-0003.

Riu A, Grimaldi M, le Maire A, Bey G, Phillips K, Boulahtouf A, et al. 2011a. Peroxisome proliferator-activated receptor γ is a target for halogenated analogs of bisphenol A. Environ. Health Perspect. 119:1227–1232; doi:10.1289/ehp.1003328.

Riu A, le Maire A, Grimaldi M, Audebert M, Hillenweck A, Bourguet W, et al. 2011b. Characterization of novel ligands of ERα, ERβ, and PPARγ: The case of halogenated bisphenol A and their conjugated metabolites. Toxicol. Sci. 122:372–382; doi:10.1093/toxsci/kfr132.

Rochester JR. 2013. Bisphenol A and human health: A review of the literature. Reprod. Toxicol. 42:132–155; doi:10.1016/j.reprotox.2013.08.008.

Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. 1998. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicol. Appl. Pharmacol. 153:12–9; doi:10.1006/taap.1998.8544.

Ruiz-Cortés ZT. 2012. Gonadal sex steroids: Production, action and interactions in mammals. In *Steroids - From Physiology to Clinical Medicine* (S.M. Ostojic, ed.), pp. 3–44, InTech.

Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, Keri RA. 2016. A review of the carcinogenic potential of bisphenol A. Reprod. Toxicol. 59:167–182; doi:10.1016/j.reprotox.2015.09.006.

Shaw SD, Harris JH, Berger ML, Subedi B, Kannan K. 2014. Brominated flame retardants and their replacements in food packaging and household products: Uses, human exposure, and health effects. In *Toxicants in Food Packaging and Household Plastics: Exposure and Health Risks to Consumers* (S. Snedeker, ed.), pp. 61–93, Springer, London.

Sieli PT, Jašarevic E, Warzak DA, Mao J, Ellersieck MR, Liao C, et al. 2011. Comparison of serum bisphenol A concentrations in mice exposed to bisphenol A through the diet versus oral bolus exposure. Environ. Health Perspect. 119: 1260–1265.

Stoker TE, Gibson EK, Zorrilla LM. 2010. Triclosan exposure modulates estrogen-dependent responses in the female Wistar rat. Toxicol. Sci. 117:45–53; doi:10.1093/toxsci/kfq180.

Suzuki G, Tue NM, Malarvannan G, Sudaryanto A, Takahashi S, Tanabe S, et al. 2013. Similarities in the endocrine-disrupting potencies of indoor dust and flame retardants by using human osteosarcoma (U2OS) cell-based reporter gene assays. Environ. Sci. Technol. 47:2898–2908; doi:10.1021/es304691a.

Takeuchi S, Iida M, Kobayashi S, Jin K, Matsuda T, Kojima H. 2005. Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. Toxicology 210:223–233; doi:10.1016/j.tox.2005.02.002.

Thomas P, Dong J. 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. J. Steroid Biochem. Mol. Biol. 102:175–179; doi:10.1016/j.jsbmb.2006.09.017.

U.S. EPA. 2017a. Collaborative Estrogen Receptor Activity Prediction Project Data. Available: https://www3.epa.gov/research/COMPTOX/CERAPP_files.html [accessed 3 May 2017].

U.S. EPA. 2017b. Endocrine Disruption: Endocrine Disruptor Screening Program (EDSP). Available: https://www.epa.gov/endocrine-disruption#universe [accessed 3 May 2017].

U.S. EPA. 2012a. Endocrine Disruptor Screening Program: Universe of Chemicals. Available: https://www.epa.gov/sites/production/files/2015-07/documents/edsp_chemical_universe_list_11_12.pdf [accessed 3 May 2017].

U.S. EPA. 2012b. Endocrine Disruptor Screening Program: Universe of Chemicals and General Validation Principles. Available: https://www.epa.gov/sites/production/files/2015-08/documents/edsp_chemical_universe_and_general_validations_white_paper_11_12.pdf [accessed 3 May 2017].

U.S. EPA. 1988. Integrated Risk Information System (IRIS). Chemical assessment summary for bisphenol A (CASRN 80-05-7). Available: https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0356_summary.pdf [accessed 18 April 2017].

Valbuena D, Martin J, De Pablo JL, Remohí J, Pellicer A, Simón C. 2001. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. Fertil. Steril. 76:962–968; doi:10.1016/S0015-0282(01)02018-0.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, et al. 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33:378–455; doi:10.1210/er.2011-1050.

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. 2007. Human exposure to bisphenol A (BPA). Reprod. Toxicol. 24:139–177; doi:10.1016/j.reprotox.2007.07.010.

vom Saal FS, Hughes C. 2005. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ. Health Perspect. 113:926–933; doi:10.1289/ehp.7713.

vom Saal FS, Welshons W V. 2006. Large effects from small exposures. II. The importance of positive controls in low-dose research on bisphenol A. Environ. Res. 100:50–76; doi:10.1016/j.envres.2005.09.001.

Walters MR. 1985. Steroid hormone receptors and the nucleus. Endocr. Rev. 6: 512–543.

Wang L-Q, Falany CN, James MO. 2004. Triclosan as a substrate and inhibitor of 3′-phosphoadenosine 5′-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. Pharmacology 32:1162–1169; doi:10.1124/dmd.104.000273.the.

Wang L, Asimakopoulos AG, Kannan K. 2015. Accumulation of 19 environmental phenolic and xenobiotic heterocyclic aromatic compounds in human adipose tissue. Environ. Int. 78:45–50; doi:10.1016/j.envint.2015.02.015.

Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y, et al. 2012. Urinary bisphenol A (BPA) concentration associates with obesity and insulin resistance. J. Clin. Endocrinol. Metab. 97:223–227; doi:10.1210/jc.2011-1989.

Watanabe Y, Kojima H, Takeuchi S, Uramaru N, Ohta S, Kitamura S. 2013. Comparative study on transcriptional activity of 17 parabens mediated by estrogen receptor α and β and androgen receptor. Food Chem. Toxicol. 57:227–234; doi:10.1016/j.fct.2013.03.036.

Welshons WV., Nagel SC, Vom Saal FS. 2006. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 147:56–69; doi:10.1210/en.2005-1159.

Welshons WV., Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ. Health Perspect. 111:994–1006; doi:10.1289/ehp.5494.

White PC. 1994. Genetic diseases of steroid metabolism. Vitam. Horm. 49: 131—195.

Wikoff DS, Rager JE, Haws LC, Borghoff SJ. 2016. A high dose mode of action for tetrabromobisphenol A-induced uterine adenocarcinomas in Wistar Han rats: A critical evaluation of key events in an adverse outcome pathway framework. Regul. Toxicol. Pharmacol. 77:143–159; doi:10.1016/j.yrtph.2016.01.018.

Ye X, Bishop AM, Needham LL, Calafat AM. 2008a. Automated on-line column-switching HPLC-MS/MS method with peak focusing for measuring parabens, triclosan, and other environmental phenols in human milk. Anal. Chim. Acta 622:150–156; doi:10.1016/j.aca.2008.05.068.

Ye X, Tao LJ, Needham LL, Calafat AM. 2008b. Automated on-line column-switching HPLC-MS/MS method for measuring environmental phenols and parabens in serum. Talanta 76:865–871; doi:10.1016/j.talanta.2008.04.034.

Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB. 1998. Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. Toxicol. Sci. 46:282–293; doi:10.1006/toxs.1998.2505.

Zalko D, Prouillac C, Riu A, Perdu E, Dolo L, Jouanin I, et al. 2006. Biotransformation of the flame retardant tetrabromo-bisphenol A by human and rat sub-cellular liver fractions. Chemosphere 64:318–327; doi:10.1016/j.chemosphere.2005.12.053.

Zarean M, Keikha M, Poursafa P, Khalighinejad P, Amin M, Kelishadi R. 2016. A systematic review on the adverse health effects of di-2-ethylhexyl phthalate. Environ. Sci. Pollut. Res. 23:24642–24693; doi:10.1007/s11356-016-7648-3.

Zhang L, Ding S, Qiao P, Dong L, Yu M, Wang C, et al. 2016. *n*-Butylparaben induces male reproductive disorders via regulation of estradiol and estrogen receptors. J. Appl. Toxicol. 36:1223–1234; doi:10.1002/jat.3291.

Ziv-Gal A, Flaws JA. 2016. Evidence for bisphenol A-induced female infertility: A review (2007–2016). Fertil. Steril. 106:827–856; doi:10.1016/j.fertnstert.2016.06.027.

Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, et al. 2012. Endocrine-disrupting chemicals and public health protection: A statement of principles from the Endocrine Society. Endocrinology 153:4097–4110; doi:10.1210/en.2012-1422.

Zota AR, Calafat AM, Woodruff TJ. 2014. Temporal trends in phthalate exposures: Findings from the national health and nutrition examination survey, 2001-2010. Environ. Health Perspect. 122:235–241; doi:10.1289/ehp.1306681.