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PREGNANCY DISRUPTION BY TRICLOSAN ALONE AND WITH BISPHENOL-A

DISRUPTION OF EARLY PREGNANCY IN THE CF-1 MOUSE: IMPACTS OF TRICLOSAN ALONE AND IN COMBINATION WITH BISPHENOL-A

By BRENT R. CRAWFORD, B.SC.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

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McMaster University MASTER OF SCIENCE (2012) Hamilton, Ontario (Psychology, Neuroscience & Behaviour)

TITLE: Disruption of Early Pregnancy in the CF-1 Mouse: Impacts of Triclosan Alone and in Combination with Bisphenol-A AUTHOUR: Brent Crawford, B.Sc. (McMaster University) SUPERVISOR: Professor D. deCatanzaro NUMBER OF PAGES: ix, 39

Abstract

Triclosan is an antimicrobial additive found in a number of personal care and household products. Widely detected in humans, the compound has been given increasing attention due to reports of its endocrine-disrupting potential. Recent evidence indicates that triclosan is mildly estrogenic. The carefully timed event of blastocyst implantation in mammals is modulated in part by estrogen and can be disrupted by above optimal elevations in estrogenic stimulation. Here, we examined the influences of triclosan administration in inseminated female mice. Doses of 18 and 27 mg/animal/day on gestation days (GD) 1–3 reduced implantation site numbers as observed on GD 6, relative to vehicle controls and females given lower doses. Single doses of 18 or 27 mg reduced implantations when given on GD 3, whereas only 27 mg did so when given on GD 2. Subsequently, we examined the impacts on early pregnancy of triclosan in combination with the xenoestrogen bisphenol-A, which has been previously found to disrupt implantation, at doses that were individually ineffective. A combination of 4 mg BPA and 9 mg triclosan/animal/day administered on GD 1–3 reduced the number of implantations observed on GD 6 and increased the length of gestation, relative to controls and those animals simply given one or the other compound. All of these effects mimicked stronger effects seen in positive controls given 17β-estradiol. These data are consistent with the notion that triclosan has mild estrogenic properties, and show that it can act together with a known xenoestrogen to disrupt implantation.

Acknowledgements

I would first like to acknowledge and thank Dr. Denys deCatanzaro. I feel truly fortunate to have met and worked with a supervisor who is not only a great scientist, but also a wonderful mentor, confidant, and friend. Denys, you gave me the freedom to work and learn independently, were there whenever I needed to chat about research goals or life in general, and have been supportive even at times when I did not fully believe in myself. Thank you for guiding me successfully through the past two years. I would also like to thank my committee members, Drs. Jeff Galef and Reuven Dukas, for their continued advice and words of encouragement. To Adam Guzzo and Joelle Thorpe, thank you for being there to lend a helping hand and to provide a well-needed laugh. I could not have asked for better senior lab-mates. Nazanin Rajabi, it only took us labelling a batch of urine-collection tubes one summer day to become the best of buds. The past two years would not have been the same without you. Thank you to past lab members Jihwan Jheon and Faizan Imtiaz (or two of the three amigos) for your assistance with tasks related to the project and for making the conducting of an undergraduate thesis a thoroughly entertaining process. Special thanks go to Iris Kwok for your commitment to the project this past year. You put in those long hours any day of the week and any time of the day. Congratulations on completing your undergraduate degree and best of luck in your future endeavours. Finally, I would like to thank those family members and friends who kept me emotionally sound and able to complete yet another degree. Your support was endless and you are all in part responsible for any of my successes.

Table of Contents

Title Page i

Descriptive Note ii

Abstract iii

Acknowledgements iv

List of Figures vi

List of Abbreviations viii

Declaration of Academic Achievement ix

Introduction 1

Methods 6

Results 10

Discussion 24

Bibliography 31

List of Figures

Figure 1: Mean (± SE) numbers of implantation sites counted GD 6 per inseminated female after repeated subcutaneous injections of triclosan on GD 1–3. (*) Denotes a significant difference from the vehicle control group ($p < 0.05$). (**) Denotes a significant difference from the vehicle control group ($p < 0.01$)..... 11

Figure 2: Mean (± SE) numbers of implantation sites counted on GD 6 per inseminated female after a single subcutaneous injection of triclosan or estradiol (E2) on either of GD 0, 1, 2, or 3. (*) Denotes a significant difference from the vehicle control group of the respective day ($p < 0.05$). (**) Denotes a significant difference from the vehicle control group of the respective day ($p < 0.01$). (‡) Denotes a significant difference from the vehicle control group of every other day ($p < 0.01$)..... 14

Figure 3: Mean (± SE) numbers of implantation sites counted on GD 6 per inseminated female after repeated subcutaneous injections of triclosan (TCS), BPA, estradiol (E2), or TCS and BPA on GD 1–3. (*) Denotes a significant difference from the vehicle control and 9 TCS groups ($p < 0.05$). (***) Denotes a significant difference from all other treatment groups with the exception of the other E2 positive control ($p < 0.001$)..... 18

Figure 4: The percentages of inseminated female mice with normally-developing implantation sites observed on GD 6 after repeated subcutaneous injections of triclosan (TCS), BPA, estradiol (E2), or TCS and BPA on GD 1–3. (*) Denotes a significant difference between two groups connected by a line ($p < 0.05$). (**) Denotes a significant difference from the vehicle control group ($p < 0.01$). (***) Denotes a significant difference from the vehicle control group ($p < 0.001$). (‡) Denotes a significant difference between two groups connected by a line ($p < 0.01$)..... 19

Figure 5: Representative GD 6 uteri with attached oviducts and ovaries from inseminated CF-1 female mice treated with BPA, triclosan, estradiol, or BPA and triclosan during the pre-implantation period. (A) Normally-developing: Uterus with normally-developing, well-defined implantation sites (arrows). (B) Underdeveloped: Uterus with uncharacteristically small or

underdeveloped implantation sites that are not easily discriminated visually as protuberances in otherwise smooth uterine horns (arrows). (C) Non-implanted: Uterus with no implantation sites..... 20

Figure 6: The mean (± SE) gestation lengths of inseminated female mice after repeated subcutaneous injections of triclosan (TCS), BPA, estradiol (E2), or TCS and BPA on GD 1–3. (***) Denotes a significant difference from the vehicle control, 9 mg TCS, 2 mg BPA, 4 mg BPA, and 2 mg BPA + 9 mg TCS groups ($p < 0.001$)……... 23

List of Abbreviations

Gestation Day	GD
Bisphenol-A	BPA
Post-Natal Day	PND

Declaration of Academic Achievement

The contents of this thesis were contributed to by Brent Crawford, in partial fulfillment of the requirements for the degree Master of Science, who consulted with Dr. Denys deCatanzaro. All experiments were designed by Dr. deCatanzaro and Brent Crawford, and were conducted by Brent Crawford. Experiment 1 was conducted in partial fulfillment of the requirements for the degree Honours Bachelor of Science at McMaster University conferred in June, 2011. The results and interpretation of that experiment are included in this thesis for logical flow, as Experiments 2–4 are follow-up experiments to Experiment 1.

Introduction

Triclosan is an antimicrobial agent used in consumer products including soaps, surface cleaners, hair products, skin cleansers and moisturizers, toothpastes, mouthwashes, deodorants, fabrics, and children's toys (see Rodricks et al., 2010; Fang et al., 2010; Dann & Hontela, 2011). The compound goes by several other names including Irgasan, Ster-Zac, Lexol 300, Cloxifenolum, Microban, and Biofresh, among others. Its use is relatively unrestricted, with concentrations up to 0.3% typically permitted in personal care products (Bedoux et al., 2012). Triclosan is readily absorbed through skin (Moss et al., 2000; Queckenberg et al., 2010) as well as the gastrointestinal tract following oral ingestion (Sandborgh-Englund et al., 2006), and one study places the highest estimated oral exposure to triclosan at 0.13 mg/kg/day (Rotroff et al., 2010). A number of reports have found triclosan in human plasma at 0.01–38 μg/kg (Allmyr et al., 2006; Dirtu et al., 2008), breast milk at 0–2100 μg/kg (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Dayan, 2007; Azzouz et al., 2011; Toms et al., 2011), and urine at 0.18–3790 μg/L (Calafat et al., 2008; Geens et al., 2009; Kim et al., 2011). It has also been measured in adipose, brain, and liver tissues up to 3.92, 0.23, and 29.03 μg/kg, respectively (Geens et al., 2012). After triclosan-containing products are used they are largely rinsed down the drain and transported to wastewater treatment plants, where the compound is incompletely removed. Remaining triclosan enters the aquatic environment, with treatment plant effluent samples showing 0.01–5.37 μg/L (McAvoy et al., 2002; Sabaliunas et al., 2003; Lishman et al., 2006; Coogan et al., 2007; Ying & Kookana, 2007; Kumar et al., 2010). Triclosan is the one of the more frequently detected and highly

concentrated surface water contaminants (Kolpin et al., 2002), and it has been detected in bottled and tap water at concentrations up to 0.1 and 0.14 μg/L, respectively (Li et al., 2010).

Triclosan raises concern as an endocrine disruptor with potential estrogenic and/or androgenic effects. Early reports in fish showed fin length and sex ratio trends that suggested weakly androgenic action (Foran et al., 2000). Conversely, male fish were observed to have decreased sperm counts and induction of normally female-limited vitellogenin expression, which is estrogen-dependent and a well-established biomarker of exposure to environmental estrogens (Ishibashi et al., 2004; Raut & Angus, 2010). In adult male rats, oral doses of 5, 10, or 20 mg triclosan/kg/day for 60 days were reported to decrease serum testosterone, sperm production, and masses of testes and male accessory glands (Kumar et al., 2009). In immature male rats, oral triclosan doses up to 300 mg/kg administered from post-natal days (PND) 23 to 53 did not alter the onset of preputial separation, a marker of male pubertal development, and serum testosterone was decreased at 200 mg/kg only (Zorilla et al., 2009). In female rats, oral triclosan administration from PND 19 to 21 at doses of 7.4, 37.4, and 187.5 mg/kg/day significantly increased uterine weight and the expression of genes upregulated by natural estrogens (Jung et al., 2012). Elsewhere (Rodriguez & Sanchez, 2010; Stoker et al., 2010), similar administration of triclosan up to 300 mg/kg/day was insufficient to increase the uterine weight of immature females alone; however, doses of 4.69 mg triclosan/kg/day and higher were able to modulate the effect of ethinyl estradiol on uterine growth in a dose-dependent manner (Stoker et al., 2010).

The period between fertilization and intrauterine implantation of fertilized ova is highly sensitive to fluctuations in natural estrogens. In a careful timeframe, estrogen is in part responsible for preparing both the developing embryo and the uterine environment for implantation (Roblero & Garavagno, 1979; Paria et al., 1993; Potter et al., 1996; Wang & Dey, 2006). Supraphysiological estrogenic stimulation can disrupt this interaction by altering uterine integrity (Ma et al., 2003), embryo development (Valbuena et al., 2001), and transport from the oviduct (Burdick & Whitney, 1937; Greenwald, 1967). For example, peri-implantation injections of as little as 37 ng/animal/day of 17β-estradiol can terminate pregnancy (deCatanzaro et al., 1991, 2001). Injections of the xenoestrogen, bisphenol-A (BPA), can similarly impact blastocyst implantation in doses of 6.75 or 10.125 mg/animal/day on gestation days (GD) 1–4 (Berger et al., 2007, 2008, 2010). Single injections of 6.75 mg on day 1, or 10.125 mg on either of GD 0 or 1 could also significantly reduce the number of implantation sites observed on GD 6 (Berger et al., 2008). Additionally, repeated pre-implantation exposure to BPA was shown to alter uterine morphology, where doses of 6.75 or 10.125 mg BPA/animal/day significantly expanded observed luminal areas, and exposure to 10.125 mg increased epithelial cell heights (Berger et al., 2010).

Accordingly, we first administered triclosan to inseminated mice to determine whether the compound could disrupt early pregnancy. To our knowledge, effects of triclosan upon implantation have not been systematically examined; however, several studies have investigated gestational exposure to triclosan in rodents. Two recent studies, which primarily focused on potential thyroid disruption, incidentally showed no effect of

oral administration up to 300 mg/kg/day (Paul et al., 2010) and up to 50 mg/kg/day (Rodriguez & Sanchez, 2010) on gestation length, pregnancy rate, or litter size. Rodricks et al. (2010) reviewed several reproductive and teratological reports by a pharmaceutical company showing similarly negative results. The exposure regimens for all of these studies either began after implantation or spanned from at least one week prior to mating until pup weaning, and thus only limited conclusions can be formed concerning implantation *per se*. In the present study, females were subcutaneously injected from GD 1–3, timing that corresponds to the pre-implantation period in mice (Paria et al., 1993), and examined on GD 6 for intrauterine implantation sites. In a follow-up experiment, doses that significantly reduced the number of implantations after repeated administration were tested for impact on pregnancy after a single exposure on either of GD 0, 1, 2, or 3.

Subsequently, we investigated whether triclosan could act in binary mixtures with BPA to perturb early pregnancy. Traditionally, assessments for chemicals of concern are performed on an individual basis, oftentimes finding lowest observable doses of impact that are several orders of magnitude higher than those present in the environment, which leads to conclusions that any risks posed are negligible. This approach does not take into account the fact that humans and wildlife are exposed to a variety of endocrine-disrupting chemicals that have similar mechanisms of action or affect similar endpoints. Increasing attention is being given to the nature of combination effects of endocrine disruptors (Kortenkamp, 2007). Combinations of estrogenic compounds at low, environmentally-relevant, and individually ineffective levels have been shown to have impact together *in vitro* (Rajapakse et al., 2002; Silva et al., 2002; Charles et al., 2007; Silva et al., 2011)

and *in vivo* using fish (Thorpe et al., 2001; Brian et al., 2005, 2007; Jin et al., 2012) and rat models (Tinwell & Ashby, 2005; Charles et al., 2007). We administered triclosan in combination with BPA to recently inseminated female mice, using doses that were below the thresholds necessary for each substance to disrupt implantation on its own (*cf*. Berger et al., 2007, 2008, 2010).

Methods

Animals and housing

This research was approved by the Animal Research Ethics Board of McMaster University in compliance with the guidelines of the Canadian Council on Animal Care. CF-1 mice (*Mus musculus*) were of stock from Charles River Breeding Farms of Canada (La Prairie, Québec). Mice were housed in standard 28 cm x 16 cm x 11 cm (height) polypropylene cages, with *ad libitum* access to food (8640 Teklad Certified Rodent chow; Harlan Teklad, Madison, Wisconsin) and water. Colony rooms were maintained at 21 ^{o}C with a reversed 14:10 h light:dark cycle. Sexually naïve female mice aged 3-6 months were each randomly paired with a CF-1 male aged 3-6 months. Female hindquarters were inspected three times per day during the dark phase of the light cycle for the presence of vaginal sperm plugs. The date of a plug was designated as GD 0. Females were pseudo-randomly assigned to one of the experimental conditions with age and weight counterbalanced. On GD 1, each inseminated subject female was housed alone in a clean cage with fresh bedding.

Experiment 1: Repeated triclosan administration

Subcutaneous injections of triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol; >97%, Sigma-Aldrich) dissolved in peanut oil were administered approximately 4-6 h into the dark phase of the light cycle on GD 1–3. Females were assigned to doses of 0, 3, 9, 18, or 27 mg/animal/day (about 0, 90, 275, 550, or 825 mg/kg/day), with sample sizes of 31, 13, 13, 13, and 21, respectively. The mean (±SE) mass of subjects was 34.4±0.4 g at GD 1. Triclosan doses of 3, 9, and 18 mg were dissolved in 0.05 ml oil, whereas the 27

mg dose was dissolved in 0.10 ml oil due to solubility constraints. Proportional numbers of control (0 mg) subjects were run at these volumes of oil. Different quantities of vehicle did not have an impact on pregnancy outcome in these controls. To minimize any irritation caused by administration of triclosan, injections occurred at three different sites: right flank, left flank, and the scruff of the neck. Experimental and control subjects were given injections in identical locations. Pregnancy outcome was measured about 4-6 h after commencement of the dark phase of the light cycle on GD 6. Females were sacrificed by cervical dislocation after 2 min of isoflurane anesthetic. Their uteri were excised via abdominal incision and the number of implantation sites was counted. An implantation site was defined as a round protuberance in an otherwise smooth and uninterrupted uterine horn.

Experiment 2: Single dose triclosan administration by day of gestation

A single subcutaneous injection of triclosan dissolved in oil was administered in the scruff of the neck on either GD 0 at the time of sperm plug detection, counterbalancing for time across groups, or on GD 1, 2, or 3 at 4-6 h after start of the dark phase of the light cycle. The mean mass of subjects was 31.3±0.4 g. Females were assigned to doses of 0, 18, or 27 mg triclosan/animal, or to doses of 100 or 200 ng/animal (about 0.003 or 0.006 mg/kg/animal) 17β-estradiol (>98%, Sigma) to provide positive controls. Sample sizes were, respectively, 20, 9, 9, 9, and 10 for day 0; 20, 9, 9, 9, and 10 for day 1; 19, 8, 15, 9, and 10 for day 2; and 10, 10, 15, 9, and 10 for day 3. Doses were dissolved in 0.05 ml oil, except for the 27 mg triclosan doses, which were dissolved in

0.1 ml oil. A proportional number of 0 mg controls were run with 0.1 ml oil. Pregnancy outcome measurement was as in Experiment 1.

Experiment 3: Combinations of repeated sub-threshold BPA and triclosan doses

Subcutaneous injections of BPA (97%, Sigma-Aldrich) and/or triclosan were administered on GD 1-3 following procedures of Experiment 1. The mean mass of subjects was 32.5±0.3 g at GD 1. Females were assigned to one of the following dosage groups, each administered per animal per day: 2 mg BPA (about 60 mg/kg), 4 mg BPA (about 120 mg/kg), 9 mg triclosan, 2 mg BPA + 9 mg triclosan, 4 mg BPA + 9 mg triclosan, a vehicle control, or the positive control groups of 100 or 200 ng 17β-estradiol. Sample sizes were 11, 15, 12, 14, 16, 17, 7, and 7, respectively. Due to solubility constraints, triclosan, BPA, and estradiol doses were dissolved in different volumes of oil: estradiol in 0.05 ml; 2 mg BPA and 9 mg triclosan in 0.1 ml; and 4 mg BPA in 0.2 ml. Every animal received two injections on each day, with a total injection volume of either 0.2 or 0.3 ml. To minimize irritation, injections occurred at 6 different sites: twice in the right flank, twice in the left flank, once in the middle back area, and once in the scruff of the neck, with all subjects given injections in the same pattern. Pregnancy outcome measurement was carried out as in Experiment 1.

Experiment 4: Impact from birth to weaning of combinations of BPA and triclosan

Additional samples of inseminated females were administered subcutaneous injections of BPA, triclosan, and estradiol dissolved in oil on GD 1-3 in the same conditions as in Experiment 3. Sample sizes were 10, 8, 10, 10, 9, 16, 7, and 7, respectively. The mean mass of subjects was 31.9±0.3 g at GD 1. Commencing on GD 17

and continuing until 25 days after sperm plug detection, females were monitored for parturition three times per day. The date of parturition was designated as PND 0. The number of pups and the body weights of each pup were monitored from birth to weaning on PND 0, 4, 7, 14, and 21. On PND 21, the sex ratio of pups was determined.

Statistical analysis

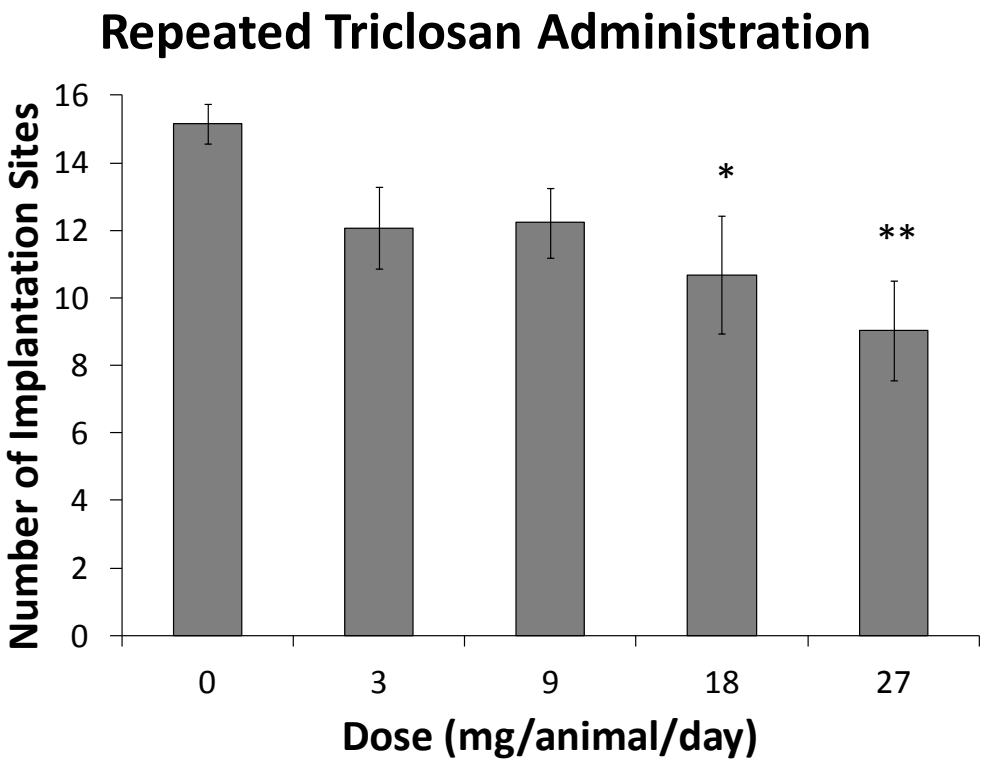
A one-way analysis of variance was used to examine the effect of dose group on the quantitative measures examined in Experiments 1, 3, and 4, including number of implantation sites, litter size, gestation length, and post-natal survival. Where significance was observed, the Newman-Keuls procedure was implemented to examine all multiple pair-wise comparisons. For Experiment 2, orthogonal *t*-tests were conducted to compare the average number of implantation sites counted for each experimental group to the same day vehicle control group. Measures of implantation site number and litter size include zeros for non-implanted and non-parturient mice, respectively. A repeated-measures analysis of covariance was used to examine pup weight differences in Experiment 4 from birth to weaning, using corresponding litter sizes as a changing covariate. Finally, the χ^2 test was initially used to analyze the percentages of dams with normally-developing implantation sites in Experiment 3 as well as the PND 21 gender ratios in Experiment 4. If significance was observed, pair-wise comparisons were examined using Fisher's exact tests. For all analyses, results were considered to be significant at the $p < 0.05$ level.

Results

Experiment 1: Repeated triclosan administration

The average numbers of implantation sites counted on GD 6 in the uterine horns of inseminated CF-1 female mice for each triclosan dosage group are shown in Figure 1. A moderate decrease in the numbers of implantation sites observed is evident at the higher doses of 18 and 27 mg triclosan, which were administered per day from GD 1–3. A one-way analysis of variance revealed a significant effect of dosage group on the number of implantation sites, $F(4,86) = 5.31$, $p = 0.001$. Multiple comparisons demonstrated that the 18 mg triclosan group ($p < 0.05$) and the 27 mg triclosan group ($p < 0.01$) differed significantly from the vehicle control group.

Figure 1: Mean (± SE) numbers of implantation sites counted GD 6 per inseminated female after repeated subcutaneous injections of triclosan on GD 1–3. (*) Denotes a significant difference from the vehicle control group ($p < 0.05$). (**) Denotes a significant difference from the vehicle control group ($p < 0.01$).

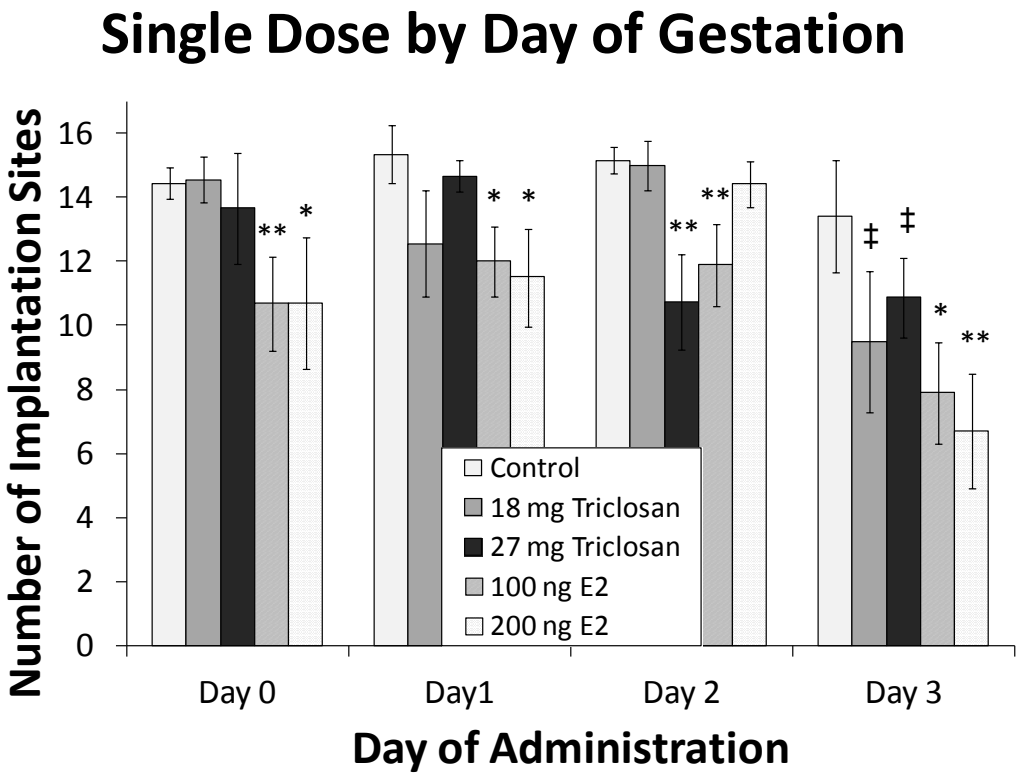


Experiment 2: Single dose triclosan administration by day of gestation

The doses of triclosan used in this experiment were chosen based on the analysis of results from Experiment 1, which indicated that repeated administration of 18 or 27 mg/animal/day had significant impact on implantation. Figure 2 illustrates the average numbers of implantation sites counted for each condition by day of subcutaneous injection. For those animals that were administered a single dose on GD 0 (i.e., immediately after the observation of a sperm plug), there was a significant implantation site number decrease in the 100 ng estradiol group, $t(27) = 3.14$, $p = 0.002$, and the 200 ng estradiol group, $t(28) = 2.38$, $p = 0.012$, compared to vehicle controls. Similarly, animals dosed on GD 1 with 100 ng estradiol, $t(27) = 2.17$, $p = 0.020$, or 200 ng estradiol, $t(28) = 2.30$, $p = 0.015$, showed a significant decrease in the numbers of implantation sites counted on GD 6, compared to vehicle controls. No significant differences were found between either of the triclosan conditions and their respective vehicle controls for those animals treated on GD 0 or 1. Among those treated on GD 2, there was a significant decrease in the numbers of observed implantation sites for 100 ng estradiol, $t(26) = 3.11$, $p = 0.002$, and 27 mg triclosan groups, $t(32) = 3.17$, $p = 0.002$, compared to vehicle controls. Finally, the average numbers of implantation sites counted were significantly lower for those animals dosed with 100 ng estradiol, $t(17) = 2.31$, $p = 0.017$, or 200 ng estradiol, $t(18) = 2.68$, $p = 0.008$, on GD 3. Although the average numbers of implantation sites did not differ between those animals administered a single dose of 18 or 27 mg triclosan on GD 3 and the vehicle control group of that day, these triclosan groups had fewer implantations than the vehicle control groups of every other day

examined. A smaller number of subjects (n=10) and one non-implanted mouse in the day 3 vehicle control group contributed to its greater implantation site number variability as compared to control groups on days 0 (n=20), 1 (n=20), and 2 (n=19). Those animals treated with 18 mg triclosan on GD 3 differed significantly in implantation site number from the vehicle control groups on day 0, $t(28) = 2.94$, $p = 0.003$, day 1, $t(28) = 2.90$, $p = 0.004$, and day 2, $t(27) = 3.38$, $p = 0.001$. Similarly, the day 3, 27 mg triclosan group differed significantly in implantation site number from the vehicle control groups on day 0, $t(33) = 2.95$, $p = 0.003$, day 1, $t(33) = 2.97$, $p = 0.003$, and day 2, $t(32) = 3.58$, $p = 0.001$.

Figure 2: Mean (± SE) numbers of implantation sites counted on GD 6 per inseminated female after a single subcutaneous injection of triclosan or estradiol (E2) on either of GD 0, 1, 2, or 3. (*) Denotes a significant difference from the vehicle control group of the respective day ($p < 0.05$). (**) Denotes a significant difference from the vehicle control group of the respective day ($p < 0.01$). (‡) Denotes a significant difference from the vehicle control group of every other day ($p < 0.01$).



Experiment 3: Combinations of repeated sub-threshold BPA and triclosan doses

After determining that triclosan could perturb implantation in inseminated female mice, we explored the potential impacts that this endocrine-disrupting substance could have in combination with BPA at individually ineffective doses. The dose of triclosan used in this experiment was chosen based on the analysis of results from Experiment 1, which indicated that repeated administration of 9 mg/animal/day was the highest dose tested that did not have a significant impact on implantation. Similarly, the sub-threshold doses of 2 mg and 4 mg BPA/animal/day were chosen based on previous evidence that demonstrated a significant impact of 6.75 mg but not 3.375 mg BPA/animal/day on the number of implantation sites observed at GD 6 in CF-1 females repeatedly administered with subcutaneous doses of on GD 1–4 (*cf*. Berger et al., 2007, 2008, 2010). The average numbers of implantation sites counted on GD 6 for each condition are shown in Figure 3. There was a dramatic decrease in the average numbers of implantation sites observed for both estradiol positive control groups, and a moderate decrease in the average number of implantation sites observed for the group receiving doses of 4 mg BPA + 9 mg triclosan from GD 1–3. The one-way analysis of variance revealed a significant effect of dosage group on the number of implantation sties observed, $F(7,91) = 15.48$, $p < 0.001$. Multiple comparisons revealed that both the 100 ng and 200 ng estradiol groups differed significantly from all other treatment groups ($p < 0.001$), but not each other. Furthermore, the 4 mg BPA + 9 mg triclosan condition differed significantly from both the vehicle control and 9 triclosan conditions ($p < 0.05$).

In addition to the effect of dosage group on implantation site number, a number of groups were observed to have dams with uteri that contained uncharacteristically small implantation sites at GD 6. For what seemed to be an all-or-none effect with regard to the implantation sites in a given uterus, each dam was identified as having normally-developing implantation sites or underdeveloped implantation sites. Underdeveloped implantation sites were those that could not be easily discriminated visually as a protuberance in an otherwise smooth uterine horn, but instead had to be felt with forceps. The percentages of dams with normally-developing implantation sites across all conditions tested are reported in Figure 4, and representative uteri are shown in Figure 5. Non-implanted mice are included in this measure, and are identified as having underdeveloped implantation sites. A χ^2 test comparing the proportions of normally-developing implantation sites across conditions was significant, $\chi^2(7) = 55.24$, $p < 0.001$. Pair-wise comparisons revealed that the 2 mg BPA + 9 mg triclosan ($p < 0.01$) and 4 mg BPA + 9 mg triclosan ($p < 0.001$) dosage combination groups had significantly lower percentages of dams with normally-developing implantation sites, compared to the vehicle control group, in which all dams had normally-developing implantations as observed at GD 6. This trend mimicked the effects seen in both estradiol-treated positive control groups, where all of the dams with uteri containing implantation sites had underdeveloped implantations. Furthermore, the 2 mg BPA + 9 mg triclosan combination group significantly differed from the 9 mg triclosan group ($p < 0.05$), and the 4 mg BPA + 9 mg triclosan combination group differed significantly from both the 9 mg triclosan (p

< 0.01) and 4 mg BPA groups ($p < 0.01$), with regard to the percentages of dams with normally-developing implantations.

Figure 3: Mean (± SE) numbers of implantation sites counted on GD 6 per inseminated female after repeated subcutaneous injections of triclosan (TCS), BPA, estradiol (E2), or TCS and BPA on GD 1–3. (*) Denotes a significant difference from the vehicle control and 9 TCS groups ($p < 0.05$). (***) Denotes a significant difference from all other treatment groups with the exception of the other E2 positive control ($p < 0.001$).

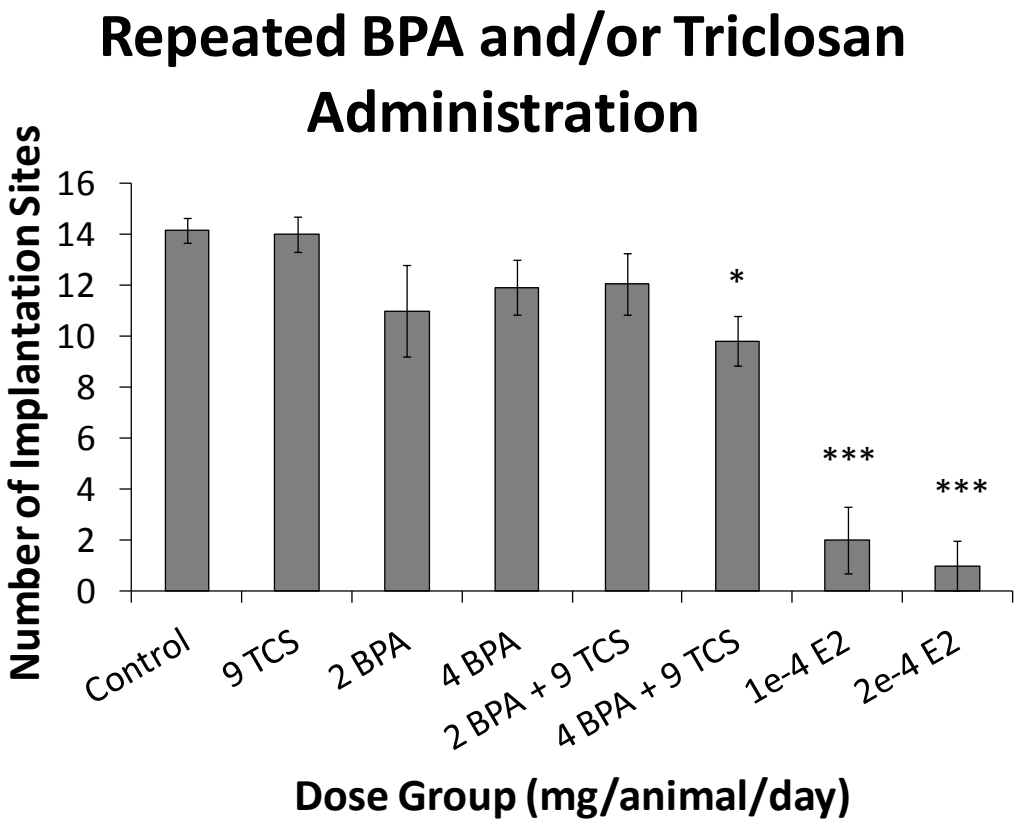


Figure 4: The percentages of inseminated female mice with normally-developing implantation sites observed on GD 6 after repeated subcutaneous injections of triclosan (TCS), BPA, estradiol (E2), or TCS and BPA on GD 1–3. (*) Denotes a significant difference between two groups connected by a line ($p < 0.05$). (**) Denotes a significant difference from the vehicle control group ($p < 0.01$). (***) Denotes a significant difference from the vehicle control group ($p < 0.001$). (‡) Denotes a significant difference between two groups connected by a line ($p < 0.01$).

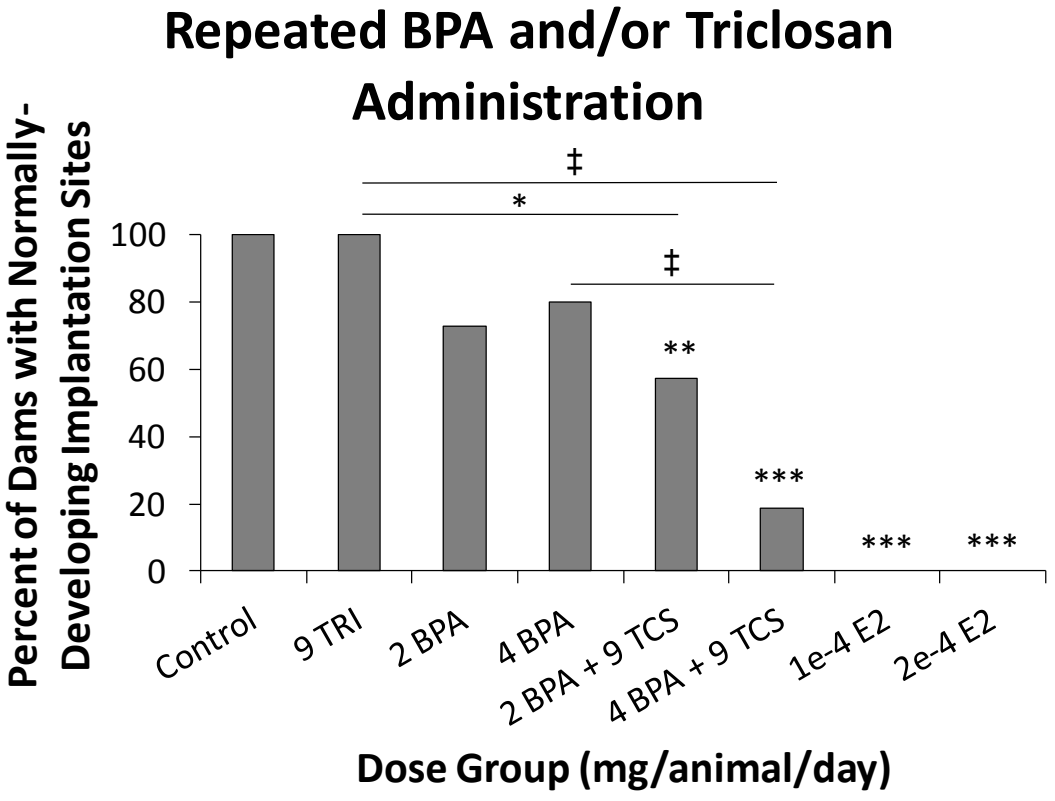
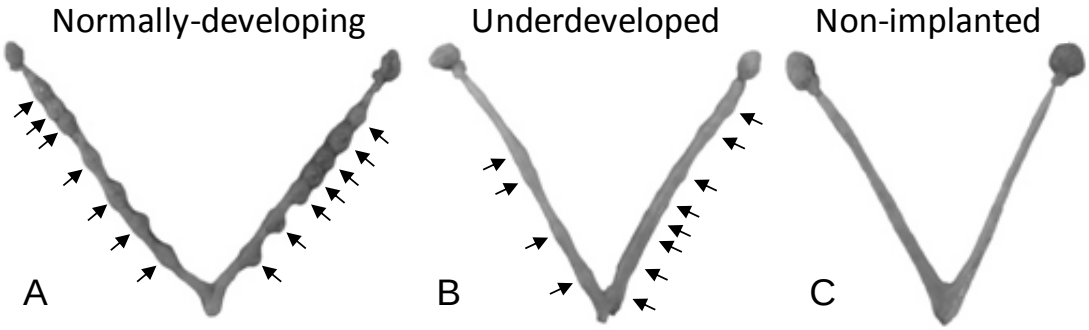


Figure 5: Representative GD 6 uteri with attached oviducts and ovaries from inseminated CF-1 female mice treated with BPA, triclosan, estradiol, or BPA and triclosan during the pre-implantation period. (A) Normally-developing: Uterus with normally-developing, well-defined implantation sites (arrows). (B) Underdeveloped: Uterus with uncharacteristically small or underdeveloped implantation sites that are not easily discriminated visually as protuberances in otherwise smooth uterine horns (arrows). (C) Non-implanted: Uterus with no implantation sites.



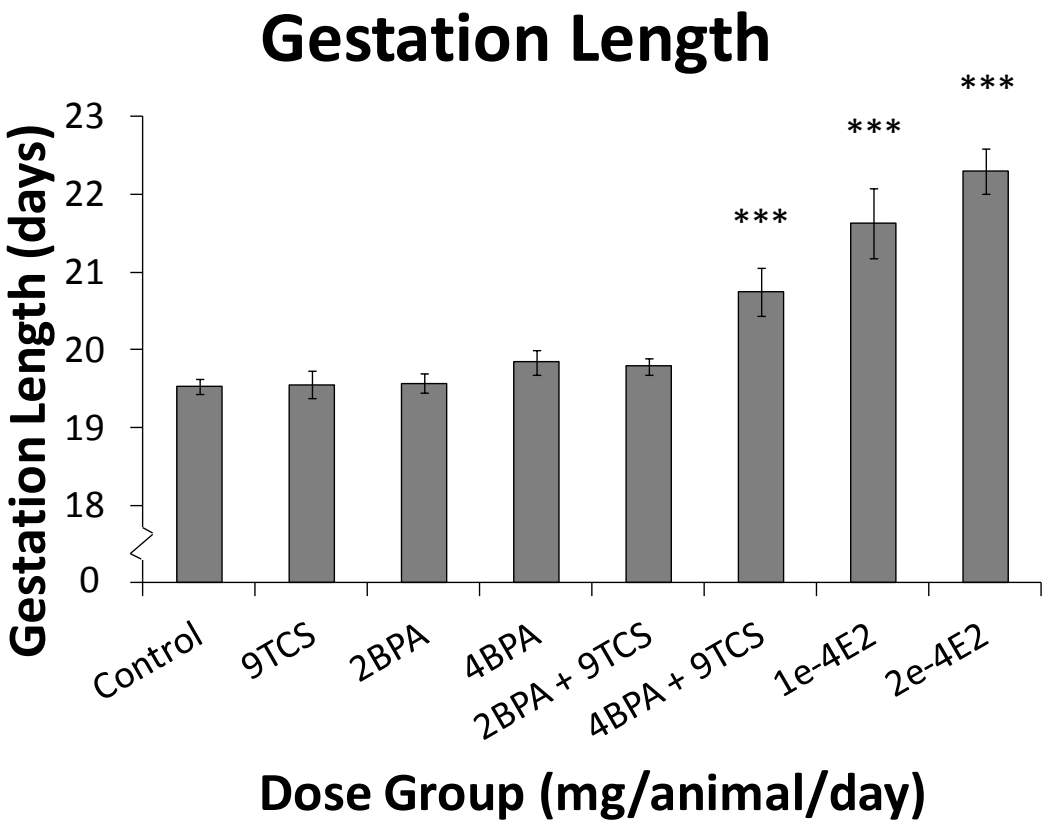
Experiment 4: Impact from birth to weaning of combinations of BPA and triclosan

Here, we explored the potential consequences associated with the observed GD 6 developmental delay of implantation sites within inseminated females treated with a combination of BPA and triclosan. The average gestation lengths of animals in all tested conditions are shown in Figure 6. There was a dramatic increase in the average length of gestation observed for both estradiol positive control groups, as well as for the group receiving doses of 4 mg BPA + 9 mg triclosan from GD 1–3. One-way analysis of variance revealed a significant effect of dosage group on gestation length, $F(7,60) = 16.28$, $p < 0.001$. Multiple comparisons demonstrated that both the 100 ng and 200 ng estradiol positive control groups differed significantly from the 4 mg BPA + 9 mg triclosan group ($p < 0.01$), as well as from all other conditions ($p < 0.001$), but not from each other. Furthermore, the 4 mg BPA + 9 mg triclosan condition differed significantly from each of the BPA and triclosan conditions, as well as the 2 mg BPA + 9 mg triclosan group and the vehicle control group ($p < 0.001$).

A one-way analysis of variance showed an effect of dosage group on PND 0 litter size, $F(7,68) = 8.51$, $p < 0.001$. Multiple comparisons revealed that this effect was attributable to the reduced numbers of pups born to animals treated on GD 1–3 with estradiol. The 100 ng estradiol positive control group differed significantly from all other conditions ($p < 0.01$), with the exception of the 200 ng estradiol positive control, which also significantly differed from every other condition ($p < 0.001$). One inseminated female from the 4 mg BPA + 9 mg triclosan condition was removed from this analysis since her litter was cannibalized before it could be counted. Although a number of

individual pups or full litters birthed from other females did not survive until weaning, this was not systematically related to dosage group. There were no significant effects of dosage group on post-natal survival, pup weights from PND 0 through PND 21, or on gender ratios (data not shown).

Figure 6: The mean (± SE) gestation lengths of inseminated female mice after repeated subcutaneous injections of triclosan (TCS), BPA, estradiol (E2), or TCS and BPA on GD 1–3. (***) Denotes a significant difference from the vehicle control, 9 mg TCS, 2 mg BPA, 4 mg BPA, and 2 mg BPA + 9 mg TCS groups ($p < 0.001$).



Discussion

These data demonstrate that high doses of triclosan have the potential to disrupt intrauterine blastocyst implantation. Experiment 1 showed that subcutaneous injections of 18 or 27 mg/animal/day of triclosan from GD 1–3 could significantly decrease the number of observed implantation sites in the uterine horns of inseminated female mice counted on GD 6 (Figure 1). Experiment 2 demonstrated a lower threshold for triclosan-induced pregnancy disruption. Females exposed to a single injection of 18 mg of triclosan on day 3 or 27 mg on either of GD 2 or 3 experienced reductions in intrauterine implantations similar to those mice that, in Experiment 1, were repeatedly administered with the same doses (Figure 2). No observable effects were elicited by doses of triclosan at 9 mg/animal/day or less. By comparison, 100 or 200 ng of estradiol was able to reduce implantation site numbers following repeated or single dose administration.

Next, in Experiment 3, we examined the potential of triclosan to act on implantation in combination with the xenoestrogen BPA at doses that were below levels of effect observed here in Experiment 1 and in previous investigations (*cf.* Berger et al., 2007, 2008, 2010). A moderate decrease in average implantation site number was observed for the group receiving doses of 4 mg BPA + 9 mg triclosan from GD 1–3 (Figure 3). Additionally, a number of groups were observed to have dams with uteri that contained uncharacteristically small implantation sites at GD 6 (Figure 5). Xiao et al. (2011) reported a similar observation in female C57BL6 mice treated from GD 0–3 with BPA, and deduced that embryo transport, development, and implantation were all being delayed by BPA administration. Accordingly, each dam in Experiment 3 was identified

as having normally-developing implantation sites or underdeveloped implantation sites. The percentages of dams with normally-developing implantations were significantly decreased in the 2 mg BPA + 9 mg triclosan and 4 mg BPA + 9 mg triclosan dosage combination groups. No significant effect was observed in those animals treated with either triclosan or BPA alone (Figure 4). Experiment 4 was conducted to determine the potential consequences associated with what seemed to be an observed implantation delay in females treated with BPA and triclosan. We found no effect of triclosan and/or BPA treatment on litter sizes, pup weights from birth through weaning, post-natal survivals, and gender ratios. The average gestation length, however, of animals receiving doses of 4 mg BPA + 9 mg triclosan from GD 1–3 after successful copulation was dramatically increased (Figure 6). Again, this trend mimicked the stronger effects seen in both estradiol-treated positive control groups, indicating that triclosan can act alone or in combination with BPA in an estrogenic fashion.

Implantation of fertilized ova into a receptive uterus is one of many critical steps in mammalian reproduction. The successful interaction between a blastocyst ready for implantation and a uterus ready to receive it is modulated in part by estrogen and can be disrupted by supraphysiological estrogenic stimulation (Burdick & Whitney, 1937; Greenwald, 1967; Roblero & Garavagno, 1979; deCatanzaro et al., 1991; Potter et al., 1996; deCatanzaro et al., 2001; Valbuena et al., 2001; Ma et al., 2003; deCatanzaro et al., 2006; Wang & Dey, 2006). Although triclosan and/or BPA was administered before the period of implantation, and acted in a fashion consistent with that of estradiol, alternative explanations of the observed results are plausible. Since triclosan has the potential to

bioaccumulate (Coogan & La Point, 2008; Fair et al., 2009), it may have lingered in the systems of treated animals and impacted the health of the dams and/or developing pups post-implantation. However, we examined implantation sites at GD 6, which makes post-implantation impacts on our results less likely. Additionally, diverse stressors have the ability to disrupt pregnancy (deCatanzaro & MacNiven, 1992). Triclosan and/or BPA administration at the doses used in this experiment, however, appeared to cause minimal irritation or discomfort to injected animals.

Initially thought to elicit effects primarily through the binding of nuclear hormone receptors, endocrine-disrupting chemicals are now understood to act through much broader mechanisms (Diamanti-Kandarakis et al., 2009). It is commonly held that BPA elicits many of its estrogenic actions through the binding of nuclear estrogen receptors α and β (Kuiper et al., 1998; Laws et al., 2000). The potential mechanisms through which triclosan can elicit its effects, however, are still largely unknown and debatable. The uncertainty stems from the sometimes conflicting research on the abilities of triclosan to impact broad endpoints associated with androgenicity (Foran et al., 2000; Christen et al., 2010), antiandrogenicity (Chen et al., 2007; Ahn et al., 2008; Gee et al., 2008; Kumar et al., 2008, 2009; Zorilla et al., 2009), estrogenicity (Stoker et al., 2010; Henry & Fair, 2011; Jung et al., 2012), antiestrogenicity (Ahn et al., 2008; Gee et al., 2008; Henry & Fair, 2011), and thyroid homeostasis (Veldhoen et al., 2006; Crofton et al., 2007; Zorilla et al., 2009; Fort et al., 2010; Paul et al., 2010; Rodriguez & Sanchez, 2010; Stoker et al., 2010). *In vitro*, triclosan appears to be able to bind to the estrogen receptors α and β as well as the androgen receptor, as evidenced by its competitive displacement of tritium-

labelled estradiol from these receptors in Michigan Cancer Foundation-7 human breast cancer cells (Gee et al., 2008). Here, however, it does not appear to behave as an agonist, but instead as a weak antagonist in the presence of natural ligands (Chen et al., 2007; Ahn et al., 2008; Gee et al., 2008; Henry & Fair, 2011), although one recent study did report estrogen-receptor-mediated transcriptional activation (Jung et al., 2012). A second way in which triclosan could be mediating estrogenic responses is through its action on estrogen metabolism. Triclosan has been shown to inhibit the estrogen sulfotransferase SULT1E1, which conjugates 17β-estradiol to estradiol-3-sulfate as part of the estrogen metabolic pathway (James et al., 2010). This disruption may consequently lead to increased levels and action of natural circulating estradiol due to under-metabolism. The metabolism of estrogen in mice, however, seems to be relatively limited compared to higher order mammals, as evidenced by low levels of estrogen conjugates present in excretions (Muir et al., 2001). This mechanism, then, may have contributed only minimally to the estrogen-mediated effects observed in this study. Furthermore, Stoker et al. (2010) demonstrated *in vivo* that triclosan administration enhanced the action of ethinyl estradiol in a dose-dependent manner on uterine growth. They proposed that triclosan could be acting as a coactivator of the receptor-ligand interaction in such a way as to enhance the steroid response (Stoker et al., 2010). In the present study, it is plausible that triclosan acted in a similar fashion to enhance the responses to BPA. We observed significant modulation of implantation site number, percentages of dams with underdeveloped implantation sites, and gestation length in those animals treated with a combination of BPA and triclosan that was not present in animals exposed to either chemical alone.

It is difficult to interpret the nature of the combined effects of triclosan and BPA on implantation in traditional terms of additivity and synergism or antagonism for a number of reasons. Additivity generally refers to the combined action of chemicals to produce effects where there is no positive (synergistic) or negative (antagonistic) interaction. Specifically, dose or concentration addition is used in the context of similarly-acting chemicals in a mixture, where any given chemical could be replaced by an equally effective portion of another chemical without altering the overall effect of the mixture (Kortenkamp, 2007). The additive relation can be expressed algebraically by the equation below (Berenbaum, 1989), where x_A and x_B represent the doses of chemicals A and B in a mixture that yields a fixed effect and X_A and X_B represent the doses of chemicals A and B alone that yield the same effect:

$$x_A/X_A + x_B/X_B = 1$$

A synergistic interaction has occurred when the left side of the equation is less than 1, indicating that the effect observed is greater than additive. Alternatively, an antagonistic interaction has occurred when the left side of the equation is greater than one, indicating that the effect observed is less than additive (Berenbaum, 1989). Firstly, the responses of implantation to triclosan and BPA separately appear to be different. Experiment 1 shows a significant decrease in implantation site number beginning at 18 mg/animal/day. At 27 mg/animal/day the response is slightly more pronounced, but overall the pregnancies of mice exposed repeatedly in the pre-implantation period seem to be inconsistently disrupted. In contrast, the disruptions of implantation by BPA exposure beginning at 6.75 mg/animal/day are relatively consistent, with most pregnancies being completely

terminated at this dose level and not below it (*cf*. Berger et al., 2008, 2010). We did not find a dose of BPA that would mimic the more moderate effect of triclosan on implantation, and as such cannot accurately interpret combined dose effects using the above model. In addition, this model assumes that the chemicals in question act through similar mechanisms of action. This might not be the case for triclosan and BPA, which elicit different responses *in vitro* (Kuiper et al., 1998; Laws et al., 2000; Chen et al., 2007; Ahn et al., 2008; Gee et al., 2008; Henry & Fair, 2011). Furthermore, when considering single-dose-administration impacts on implantation, GD 1 appears to be the most effective for BPA (*cf*. Berger et al., 2008) whereas GD 3 is the most effective for triclosan (Figure 2). This may indicate that the actions of or responses to triclosan are more immediate than those for BPA. Lastly, the doses used for Experiments 3 and 4 in this study were chosen based on the fact that these doses alone were insufficient to induce a reduction in implantation site number. We did not produce comprehensive dose-response curves for measures of endpoints like the percent of dams with normally-developing implantations or gestation length, where profound effects were found for the combination of 4 mg BPA + 9 mg triclosan. Consequently, we are not able to comment on the additive, synergistic, and/or antagonistic effects of triclosan with BPA here. The most that can be said given the present data is that triclosan appears to be contributing to the effects of BPA and/or BPA appears to be contributing to the effects of triclosan. It seems likely, however, that both chemicals yield effects that are mediated in at least some part by estrogenic action.

Overall, our data have shown that triclosan can perturb early pregnancy on its own, and that it can also do so in combination with BPA at doses below their individual sufficient doses. This contributes to the notion that these substances act via estrogen-mediated pathways. Investigations thus far regarding the safety of triclosan for use in consumer products, many of which are used on a daily basis and in combination, are limited. While some have labelled the chemical as safe at the levels to which humans are exposed (Rodricks et al., 2010), such risk assessments neglect the very real potential for combinatory effects with similarly-acting endocrine disruptors. Therefore, it seems that any absence of hazard claims may be premature. Future investigations should explore the potential of triclosan to act at lower, environmentally-relevant doses, in combination with several other xenoestrogenic compounds, employing both *in vitro* and *in vivo* paradigms.

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