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Rat strain response differences upon exposure to technical or alpha hexabromocyclododecane

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ABSTRACT

Hexabromocyclododecane (HBCD) is a brominated flame retardant which was recommended by a UN expert body under the Stockholm Convention to be eliminated from the global marketplace in 2011; however, due to its ability to persist in the environment, undergo long-range transport and bioaccumulate, it remains a concern for human health. The commercial mix of HBCD (T-HBCD) consists of α-HBCD, β-HBCD and γ-HBCD. Although the γ-HBCD (79%) isomer is the predominant isomer of T-HBCD, the most bioaccumulative isomer detected in mammals is the α-HBCD isomer. This study was undertaken to investigate three rat strains treated with commercial grade (technical) HBCD or HBCD enriched with the α isomer (A-HBCD) and to examine strain- and sex-related differences in response to exposure. Female Sprague Dawley (SD), Wistar (WI) and Fischer F344 (FI) rats were exposed for 28 days to either T-HBCD or A-HBCD in feed, at doses of 0, 250, 1250 and 5000 mg/kg diet. The FI rodent strain was found to be the most sensitive to effects of HBCD based on the greatest number of significantly affected endpoints which indicated that T-HBCD primarily affected liver and thyroid, resulting in multiple health effects. Consequently, male FI were included in the study and exposed to T- and A-HBCD. Histopathological data supports previously reported effects of HBCD on the thyroid and endocrine system although the effects in FI rats are significantly elevated compared to other strains. As with T-HBCD, liver and thyroid were found to be target organs of A-HBCD. Sex differences, specifically in tissue concentration levels, immune response parameters and in number and severity of thyroid and liver lesions, following exposure to either T- or A-HBCD were apparent, with treatment eliciting a greater response in males. Residue analysis revealed that α-HBCD is more bioaccumulative than γ-HBCD in all rodent strains, with levels of HBCD in animals treated with A-HBCD several fold higher for all tissues tested (7–11 fold at the highest dose). Thus, residue data supports the selective uptake (implies there are differences in bioavailability and/or bioaccumulation; is this the case or do certain isomers simply have a longer half-life) of specific isomers, with α-HBCD > γ-HBCD. Taken together, our study highlights the importance of selecting the most appropriate strain and of including both sexes in studies to ensure that sex-related differences in response to test chemical is taken into consideration. Moreover, ours is the first study to show the effects of a sub-acute exposure to a diet containing only HBCD enriched for the α isomer, which better represents the isomer ratios present in the biota due to bioaccumulation.

1. Introduction

The use of chemicals to impair flame propagation is not a recent phenomenon. In fact, the practice of using chemical flame retardants dates as far back as 450 BCE to when Egyptians coated wood with alum to reduce its flammability (Alaee et al., 2003). In the modern era, fire resistance of combustible materials has been increased by the application of flame retardants, including both organic and inorganic

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compounds. There are an estimated 175 flame retardants (Alaee et al., 2003; Yogui and Sericano, 2009); of these approximately 75 are brominated flame retardants (BFRs (Zhang et al., 2016);). BFRs are employed in the manufacture of consumer products to help protect from flame propagation. Hexabromocyclododecane (HBCD) has been considered a global health concern and has been shown to be environmental persistent and has the ability to bioaccumulate (Ruan et al., 2019; Zhang et al., 2009). These concerns of HBCD have led to international co-operation on chemical safety with HBCD added to Annex A of the Stockholm Convention in 2013 for elimination of use (with some exceptions) due to its classification as persistent, bioaccumulative and toxic chemical. The ban of all polybrominated diphenyl ethers (PBDEs), resulted in HBCD being the third most frequently used brominated flame retardant with an estimated worldwide annual demand of 16,700 tonnes/year (Janssen, 2005). It is most commonly employed in expandable polystyrene foam building insulation, textile coatings for upholstery in furniture, circuit boards and electronic equipment housing (Eriksson et al., 2006). Given that it is an additive flame retardant (it is blended with polymers and not covalently bound to the materials), use of products with HBCD results in greater leaching into the environment as the products are used and broken down and its continued presence in wastewater, household dust, and contaminated foods is a testament to this fact (Berger et al., 2014; Lefevre et al., 2016; Ruan et al., 2019). Based on these reasons, HBCD remains a relevant health concern.

HBCD is a brominated aliphatic cyclic hydrocarbon and the commercial formulation consists of three main diastereomers: α-, β- and γ-HBCD. HBCD has been shown to be a ubiquitous environmental contaminant that bioaccumulates in the food chain, meaning that the highest trophic levels experience the highest tissue concentrations of HBCD; thus, foods of animal origin can be an important source of exposure for humans, particularly in those whose diet consists of large amounts of fish (He et al., 2013; Schecter et al., 2008, 2012; Xian et al., 2008). Several studies have measured HBCD stereoisomers in human breast milk with mean concentrations ranging from 0.5 ng/g lipid weight (lw) – 27 ng/g lw, with Canadian samples showing a mean concentration of 3.8 ng/g lw (Eljarrat et al., 2009; Kakimoto et al., 2008; Ryan and Rawn, 2014; Ryan et al., 2006; Schecter et al., 2008). Of note, exposure through inhalation and ingestion of house dust are also considered to be important routes of HBCD exposure in young children. HBCD is found in detectable levels in both abiotic and biotic samples, including soil, atmosphere, birds, and fish as well as in human bodily fluids, including serum and milk and fetal liver and placental tissues, making it both an environmental and a human health concern (de Wit, 2002; Eldh et al., 2012; Eriksson et al., 2006; Hakk, 2016; He et al., 2013; Rawn et al., 2014a, 2014b; Ryan and Rawn, 2014).

Although HBCD has been determined to be non-genotoxic, eliciting negative results in mutagenicity and chromosomal aberration studies (Birnbaum and Staskal, 2004), other adverse effects have been observed in multiple animal models. Exposure to HBCD has been shown to cause cardiac dysfunction in zebrafish (Wu et al., 2013); increase liver weight (Chengelis, 1997; Ema et al., 2008), alter endocrine function (Chengelis, 1997, 2001; Dorosh et al., 2011; Ema et al., 2008; Fujimoto et al., 2013; Ibhazehiebo et al., 2011; Miller et al., 2016b; Tung et al., 2016; van der Ven et al., 2006, 2009; Yamada-Okabe et al., 2005), alter gestation time and sex ratio (Ema et al., 2008), decrease the number of ovarian primordial follicles in the F1 generation (Ema et al., 2008), increase thyroid weight (Chengelis, 2001; van der Ven et al., 2006), and disrupt neurodevelopment (Fujimoto et al., 2011, 2013; Saegusa et al., 2009)in rats; and alter learning and memory in mice (Eriksson et al., 2006), suggesting that HBCD is an endocrine disruptor and has multiple target organs. The available literature is both conflicting and incomplete, spurring the need for more studies on development, endocrine disruption and long-term effects of exposure to HBCD. Single oral doses of HBCD tested in rats and mice have indicated HBCD mixture-specific effects on metabolism in animals dependent on the stereoisomers present in the mixture (Szabo et al., 2011, 2017). Specifically, the potential for predicting developmental toxicity was tested in neonates (PND10) exposed to α-HBCD, γ-HBCD or the commercial mixture using serum metabolite profiles obtained 4 days post-exposure, indicating changes primarily in lipid metabolism (Szabo et al., 2017). The aim of the present study was to investigate possible strain differences in three rats strains commonly used in toxicological research (Fischer F344, Sprague Dawley and Wistar) from exposure to the commercial mixture of HBCD under identical exposure conditions. Additionally, with the propensity of α-HBCD to preferentially bioaccumulate over the other two isomers (Hakk, 2016), we also examined the effects of subacute exposure to an α-HBCD enriched mixture (A-HBCD), containing 81% α-HBCD, which has not been investigated to date.

2. Materials and methods

2.1. Animals and study design

1,2,5,6,9,10-Hexabromocyclododecane (CAS number 3194-55-6: hereafter referred to as technical HBCD or T-HBCD) 95% purity was purchased from Sigma-Aldrich (St. Louis, MO, USA). Technical HBCD consisted of the following isomer levels: α-HBCD (1%), β-HBCD (1%) and γ-HBCD (98%); the α-HBCD enriched diet (A-HBCD) consisted of the following isomer levels: α-HBCD (81%), β-HBCD (7%) and γ-HBCD (12%). A-HBCD was prepared according to the published method (Smith et al., 2005) with some modification. Briefly, technical HBCD (45 g, 70 mmol) was placed in a dry 250 ml two neck round-bottomed flask. The flask was placed in an oil bath and the mixture was heated for 1 h at 195 °C under a nitrogen atmosphere. The flask was removed from the oil bath and allowed to cool at room temperature to give hard black crude product. The product was dissolved in dichloromethane and stirred with activated charcoal then filtered and concentrated to give white crystals (MP 179–181 °C).

Rodents were fed AIN93G purified diet (Reeves, 1997). Powdered AIN93G diet was purchased from Dyets Inc. (Bethlehem, PA, USA). Treatment diets were prepared by adding powdered T-HBCD or A-HBCD to 1 kg of powdered AIN93G diet and mixing thoroughly on a roller mixer (US Stoneware, East Palestine, OH, USA) to form a homogeneous mixture. The 1 kg HBCD fortified diet was then added to 9 kg of unadulterated AIN93G and mixed for 2 h to generate the following concentrations: 250, 1250 or 5000 mg T/A-HBCD/kg diet. Control diets were prepared with unadulterated AIN93G diet. All diets were pelleted after preparation. Samples of diet (50 g) were collected upon each batch's first use and its last use and analysed for α, β and γ-HBCD as described below.

Female and male Fischer F344 (FI), and female Sprague Dawley (SD) and Wistar (WI) rats (34–37 days old) were purchased from Charles River Inc. (Montreal, QC, Canada). Upon arrival, rats were weighed prior to being housed individually in cages at 20 ± 2 °C on a 12 h light/dark cycle. Animals were acclimated for 5 days prior to start of study, during which time they were fed control diet. Throughout the study, which was approved by the Health Canada Animal Care Committee, animals were monitored and were given care and handling according to the requirements of the Canadian Council for Animal Care.

Animals were randomly assigned to one of 4 dose groups of 5 animals per group per strain. No significant differences existed in starting body weight (BW) across dose groups (Table 1). The dose levels were chosen in an attempt to produce a graded response to the test chemical. Food (control or test diet) and water were available *ad libitum* during acclimation and throughout the study treatment period. BW and food consumption (FC) were measured weekly. The first day of dosing was designated study day 0.

After 28 days of exposure following the Organization for Economic Co-Operation and Development (OECD) Test Guideline (TG) 407 study protocol (OECD, 2008), final body weights were measured on day of

Table 1
Mean daily T-HBCD consumption, initial body weights, final body weights for Fischer, Sprague Dawley and Wistar rats, for 28 days.[a] calculated from mean weekly food consumption/mean BW for each dose group over entire 28 day exposure period;[1] $p \leq 0.05$ relative to control.

	Females							
HBCD added to diet (mg T-HBCD/kg diet)	Fischer				Sprague Dawley			
	0	250	1250	5000	0	250	1250	5000
Calculated daily T-HBCD consumption[a] (mg/kg BW/day)	0.00 ± 0.00	20.16 ± 1.48	101.96 ± 11.07	429.90 ± 59.76	0.00 ± 0.00	20.70 ± 3.19	107.18 ± 15.66	411.50 ± 65.92
Initial BW (g)	119.20 ± 2.26	120.14 ± 5.95	122.48 ± 2.69	121.72 ± 5.01	155.98 ± 5.89	155.74 ± 5.61	157.32 ± 7.34	158.16 ± 7.36
Day 7 BW (g)	136.86 ± 3.79	137.86 ± 5.55	143.24 ± 4.70	144.06 ± 4.85	185.68 ± 6.81	195.88 ± 10.32	196.60 ± 11.84	194.66 ± 13.72
Day 14 BW (g)	147.94 ± 4.32	152.20 ± 5.00	**159.76 ± 7.20** [1]	**163.32 ± 7.36** [1]	211.56 ± 5.69	230.58 ± 14.39	222.46 ± 21.32	231.64 ± 17.64
Day 21 BW (g)	160.30 ± 4.10	164.36 ± 5.36	173.28 ± 10.07	**176.92 ± 8.00** [1]	230.10 ± 7.18	260.60 ± 17.66	250.66 ± 23.22	257.82 ± 21.64
Final BW (g)	167.40 ± 3.59	172.30 ± 7.31	180.32 ± 10.50	**186.92 ± 10.25** [1]	248.32 ± 7.42	283.24 ± 20.85	271.30 ± 28.92	276.10 ± 22.49
	Males							
HBCD added to diet (mg T-HBCD/kg diet)	Wistar				Fischer			
	0	250	1250	5000	0	250	1250	5000
Calculated daily T-HBCD consumption[a] (mg/kg BW/day)	0.00 ± 0.00	20.37 ± 2.81	111.62 ± 16.84	466.11 ± 76.97	0.00 ± 0.00	18.74 ± 3.34	94.12 ± 16.13	399.58 ± 77.51
Initial BW (g)	159.68 ± 5.14	157.94 ± 7.86	158.34 ± 8.34	161.10 ± 5.18	133.60 ± 4.69	134.28 ± 9.34	132.60 ± 3.84	131.52 ± 6.43
Day 7 BW (g)	194.24 ± 10.33	178.16 ± 22.57	195.30 ± 12.04	200.38 ± 7.86	169.04 ± 9.60	171.80 ± 10.75	166.56 ± 4.12	168.80 ± 7.81
Day 14 BW (g)	229.64 ± 17.98	215.48 ± 19.83	227.14 ± 17.55	243.04 ± 16.75	201.32 ± 13.11	208.72 ± 9.98	199.56 ± 3.42	204.96 ± 6.78
Day 21 BW (g)	257.68 ± 26.76	240.08 ± 17.70	251.58 ± 21.23	276.42 ± 24.19	226.88 ± 13.46	236.20 ± 10.53	226.36 ± 4.06	232.08 ± 7.68
Final BW (g)	274.42 ± 30.46	257.46 ± 17.12	275.20 ± 25.04	299.36 ± 23.89	248.16 ± 13.66	258.96 ± 10.65	246.68 ± 3.18	253.04 ± 7.95

necropsy and rats were exsanguinated via abdominal aorta under isoflurane anaesthesia. Organ weights were recorded at necropsy for liver, kidneys, thymus, spleen, brain, heart, uterus, ovaries, prostate, seminal vesicles, epididymis, testes and adrenal glands. Gross pathological assessment was also performed at necropsy. Samples of liver, serum and adipose were collected for residue determination.

2.2. Clinical observations

General health and morbidity/mortality checks were conducted cage-side twice daily throughout the study, commencing on day 0. Individual BWs were measured weekly on days 0, 7, 14, 21 and 27. FC measurements were recorded weekly on days 7, 14, 21 and 27.

2.3. Haematology and clinical chemistry

Whole blood was collected in serum separator tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) at necropsy from the abdominal aorta for haematology. Complete blood cell counts and differentials were measured using a Coulter Counter model S-PLUS IV system analyzer (Beckman Coulter Inc., Mississauga, ON, Canada). Smears for leukocyte differentials were stained with Wright's Giemsa. The following parameters were measured: white blood cells (WBCs), red blood cells (RBCs), haemoglobin (HG), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width, platelets, mean platelet volume, neutrophils, lymphocytes, monocytes, eosinophils, basophils and the ratios of each WBC type.

An aliquot of whole blood collected from each rat was centrifuged at $700 \times g$ for 20 min in serum separator tubes (Becton Dickinson) to prepare serum. All tests, with the exception of bilirubin (total and direct), creatine kinase (CK) and CO_2, which were performed on fresh serum, were conducted using serum which had been frozen at −80 °C prior to analysis. A Beckman Synchron CX5 clinical system (Beckman Instruments Canada, Inc., Mississauga) and Beckman reagent kits were used to measure clinical endpoints. The following clinical chemistry parameters were measured in the serum from each rat: albumin/globulin ratio (A/G), albumin, alkaline phosphatase (ALP), alanine transaminase (ALT), amylase, aspartate transaminase (AST), bicarbonate, bilirubin (total and conjugated), calcium (Ca), chloride (Cl), cholesterol, CK, creatinine, γ-glutamyltransferase (γ-GT), globulin, lipase, magnesium (Mg), sodium/potassium ratio (Na/K), phosphorus, potassium (K), sodium (Na), total protein, triglycerides, and urea. Total triiodothyronine (T3) and total thyroxine (T4) were measured using the Rat Thyroid 96-well plate assay (RTHY-30K; Millipore, Etobicoke, ON, Canada) according to manufacturer's instructions.

2.4. Histopathology

Gross examination of each rat was performed at necropsy. The following tissues were collected for histological examination: liver, kidney, thymus, thyroid, spleen, brain, ovaries, uterus, adrenal glands, stomach, ileum (including Peyer's patches), heart, lungs and colon. Tissues were fixed by immersion in 10% neutral buffered formalin, with the exception of testes, which were placed in Bouin's fixative. Paraffin-embedded sections (5 μm) were stained with haematoxylin and eosin (H &E), with the exception of testes and thyroid, which were stained using Periodic acid-Schiff (PAS) stain, and examined under light microscope.

2.5. Immunology

2.5.1. Flow cytometry

Changes in lymphoid cell populations in blood, spleen, mesenteric lymph nodes (MLNs) and thymus were analysed with a BD LSR Fortessa flow cytometer (BD Biosciences, Mississauga) as described previously (Curran et al., 2017).

2.5.2. T-dependent antibody response

Rats were injected intraperitoneally 4 days prior to terminal necropsy with the T-dependent antigen keyhole limpet hemocyanin (KLH; 1 mg/rat) to measure T-dependent antibody response as described previously (Curran et al., 2017).

2.5.3. Total immunoglobulin (Ig) measurement

Total IgM, IgA, and IgG in sera were measured using a sandwich ELISA according to the protocol described in Tryphonas et al. (2004). Rat IgA, IgM and IgG ELISA quantitation sets were purchased from Bethyl Laboratories, Inc. (Montgomery, Texas, USA).

2.6. Splenocyte transformation

Spleen tissue from each rat was collected at necropsy for splenocyte transformation assays. Splenocyte assays were conducted as previously described (Bondy et al., 2013).

2.7. Residue analysis

All tissues collected for residue determination were stored at −20 °C until ready for analysis. Feed samples were thawed prior to extraction and ground in 50 g aliquots, then stored frozen (−20 °C) until ready for extraction and analysis.

Prior to extraction, feed samples were thawed and thoroughly mixed manually. Approximately 0.2 g of each sample was weighed into a 15 mL polypropylene round bottomed tube, 2 mL of 1:3 (v/v) acetone:hexane (Caledon Labs, Georgetown, ON, Canada) was added to the sample and the tube was placed in an ice bath for homogenisation. Samples were homogenised for approximately 1 min using a Polytron (20,000 rpm). An additional 8 mL acetone:hexane (1:3) was added and the extract was further homogenised (5,000 rpm; 10 s). The extract was shaken on a flatbed shaker for 5 min, followed by centrifugation at 3,500 rpm ($2,800 \times g$) for 10 min. The supernatant was decanted into a solvent rinsed glass culture tube and an aliquot was transferred to a clean tube containing 25 ng $^{13}C_{12}$ α-, β- and γ-HBCD (Wellington Laboratories, Guelph, ON, Canada) and mixed using a vortex mixer, prior to further work up.

Feed, liver and serum extracts were concentrated just to dryness by placing them in a water bath set to 50 °C and placed under a gentle stream of nitrogen. Extracts were diluted to a final volume of 500 μL using methanol: water (1:1, v/v) (Caledon Labs). Prior to analysis, each sample was filtered through a 0.22 μm polyvinylidene fluoride (PVDF) filter. Samples were diluted, corresponding to the fortification level, to ensure that the concentrations in the final extracts were within the instrumental linear range.

Lipid content of samples, with the exception of serum, was determined gravimetrically. Owing to the low lipid content in serum samples, lipid determination was performed following AOAC 996.06 as previously described (Curran et al., 2017).

Following the addition of the $^{13}C_{12}$ HBCD isomers to adipose extracts, 2 mL concentrated sulphuric acid (EMD, Ottawa, ON, Canada) was added to the sample tube, gently mixed and allowed to sit for approximately 1 h. The acid layer was then removed using aspiration. Sequential aliquots of acid (2 mL) were added to samples and treated as above, until the acid remained colourless when exposed to the sample. Water (2 mL) was added and mixed with the extract. The acid and organic phases were allowed to separate, followed by removal of the aqueous layer. The extract was then passed through anhydrous sodium sulphate (Na_2SO_4) (Fisher Scientific, Ottawa, Ontario) into a clean, dry test tube and the Na_2SO_4 was rinsed with a further 1 mL of hexane and added to the cleaned up extract. The extract was then concentrated just to dryness and prepared for analysis as above.

HBCD analysis was performed using a Waters Acquity ultra

Table 2
Relative organs weights for Fischer, Sprague Dawley and Wistar rats (organ wt/BW) exposed T-HBCD.[1] $p \leq 0.05$ relative to control; ND = not determined.

	Females											
HBCD added to diet (mg T-HBCD/kg diet)	Fischer				Sprague Dawley				Wistar			
	0	250	1250	5000	0	250	1250	5000	0	250	1250	5000
Liver	3.86E-02 ± 0.16E-02	3.96E-02 ± 0.13E-02	**4.25E-02 ± 0.06E-02** [1]	**4.35E-02 ± 0.16E-02** [1]	3.92E-02 ± 0.10E-02	**4.36E-02 ± 0.14E-02** [1]	4.10E-02 ± 0.11E-02	**4.21E-02 ± 0.24E-02** [1]	3.78E-02 ± 0.24E-02	3.84E-02 ± 0.3E-02	4.23E-02 ± 0.40E-02	3.90E-02 ± 0.13-E-02
Thyroid	8.0E-05 ± 2E-05	7.7E-05 ± 2E-05	9.4E-05 ± 1E-05	10.10E-05 ± 2E-05	8.9E-05 ± 1E-05	7.90E-05 ± 1E-05	9.5E-05 ± 2E-05	10.5E-05 ± 20E-05	9.3E-05 ± 1E-05	9.5E-05 ± 2E-05	10.2E-05 ± 2E-05	10.4E-05 ± 3E-05
Thymus	2.25E-03 ± 0.3E-03	2.58 E − 03 - ± 0.3 E − 03	2.33E-03 ± 0.3E-03	2.25E-03 ± 0.1E-03	2.13E-03 ± 0.3E-03	2.51E-03 ± 0.2E-03	2.62E-03 ± 0.6E-03	2.19E-03 ± 0.3E-03	2.32E-03 ± 0.4E-03	2.60E-03 ± 0.3E-03	2.47E-03 ± 0.4E-03	2.24E-03 ± 0.3E-03
Brain	10.0E-03 ± 0.3E-03	9.5E-03 ± 0.6E-03	**9.0E-03 ± 0.2E-03** [1]	**8.9E-03 ± 0.6E-03** [1]	7.5E-03 ± 0.2E-03	6.6E-03 ± 0.4E-03	7.1E-03 ± 0.7E-03	6.9E-03 ± 0.6E-03	6.6E-03 ± 0.6E-03	7.2E-03 ± 0.6E-03	6.4E-03 ± 0.5E-03	6.0E-03 ± 0.5E-03

HBCD added to diet (mg T-HBCD/kg diet)	Males			
	Fischer			
	0	250	1250	5000
Liver	4.16E-02 ± 0.15E-02	4.18E-02 ± 0.18E-02	4.20E-02 ± 0.15E-02	4.22E-02 ± 0.14E-02
Thyroid	7.8 E − 05 ± 0.7E-05	ND	**10.9E-05 ± 0.9E-05** [1]	**10.0E-05 ± 1.4E-05** [1]
Thymus	2.02E-03 ± 0.3E-03	1.86E-03 ± 0.3E-03	2.12E-03 ± 0.1E-03	2.17E-03 ± 0.2E-03
Brain	8.2E-03 ± 0.4E-03	ND	8.2E-03 ± 0.2E-03	8.0E-03 ± 0.3E-03

performance liquid chromatograph equipped with a column heater and coupled to a Waters Quattro Premier triple quadrupole MS/MS (Waters Corporation, Milford, Massachusetts, USA) with electrospray ionization in the negative ion detection mode using a Waters BEH C18 column (2.1 mm × 100 mm, 1.7 μm) (Waters). Aqueous ammonium acetate (10 mM) (Fisher Scientific) (mobile phase A) and 10 mM ammonium acetate in methanol: acetonitrile (4:1, v/v) (mobile phase B) were used for separation of the HBCD isomers and the gradient was as follows: 30% mobile phase A initially and taken to 90% mobile phase B by 6 min where it remained until 10 min, followed by a return to the initial conditions (30% mobile phase A) by 11 min, where it remained until 14 min. The flow rate was maintained at 0.2 mL min^{-1} and the column temperature was maintained at 40 °C. The capillary and cone voltage were 1.0 kV and 15 V, respectively. The source temperature and desolvation temperature were 130 °C and 350 °C, respectively. Cone gas flow and desolvation gas flow were 30 L h^{-1} and 700 L h^{-1}, respectively. Argon was the collision gas set at 0.3 mL min^{-1} and resolution was established at 10% valley separation between adjacent masses. Dwell times were set to 5 msec. The transitions monitored for native HBCD were 641 → 79, 641 → 81and for $^{13}C_{12}$ HBCD isomers were, 653 → 79 and 653 → 81. Quantification of the unknown HBCD concentration was based on the 641 → 81 transition, with the 641 → 79 transition used for confirmation.

Reagent blanks were used to correct for any HBCD contribution due to background levels in the laboratory. A sample corresponding to the type of sample being analysed (e.g., feed, adipose, liver, serum) fortified with HBCD (2,500 μg/g γ-HBCD [feed], 250 μg/g γ-HBCD [adipose], 25 μg/g γ-HBCD [liver], 1 μg/g α-, β-, γ-HBCD [serum]) were included with each set of unknown samples and analysed for quality control purposes. The method was validated for the mean α-, β- and γ-HBCD recoveries for all tissues and various spike levels.

2.8. Benchmark dose (BMD) modelling

All study endpoints with an apparent and/or a statistically significant dose-response were modelled using the US Environmental Protection Agency's BMD software (v2.5). BMD modelling was performed as previously described (Curran et al., 2017; EPA, 2012). BMDs were derived from experimental dietary concentrations of 250, 1250, and 5000 mg HBCD/kg diet that were converted to doses listed in Tables 1 and 7 for each strain and HBCD treatment.

2.9. Ovarian follicle counts

One ovary from each female Fischer rat in the control and high dose treatment groups was embedded in paraffin, serially sectioned and stained with H&E. Follicles were identified and classified under light microscopy using a modification of Pedersen and Peters's classification system (Pedersen and Peters, 1968) as described previously (Gannon et al., 2012), with the exception that sections were 5 μm in thickness.

2.10. Statistical analysis

Statistical analyses were conducted using SigmaStat (V3.01, SPSS). Data from control and treated rats were compared using One-way analysis of variance (ANOVA) for multiple comparisons, followed by Holm-Sidak or Tukey post-hoc tests for pairwise comparisons, when necessary. Multiple group comparisons of non-parametric data were conducted using the Kruskal-Wallis ANOVA on Ranks, followed by post-hoc pairwise comparisons using the Dunn's or Tukey's method. Immunology parameters were analysed using the Pearson Product Moment Correlation or Spearman Rank Order Correlation, depending on whether or not the data were distributed normally, to measure the strength of association between dose and effect. Histopathology assessment of lesion severity data were analysed using the Cochran-Armitage trend test (2-sided Exact test p-values) to assess the probability that the severity of a given parameter would increase or decrease as the dose increases. A value of $p \leq 0.05$ was considered significant for all statistical analyses.

3. Results

3.1. Technical HBCD Study

3.1.1. Exposure to the test compound technical HBCD

Analysis of the experimental diets indicated that the achieved levels of total HBCD in T-HBCD in the diets were as follows: Control: 0.00 ± 0.00 mg/kg diet; 250 mg/kg diet: 221.26 ± 6.87 mg/kg diet; 1250 mg/kg diet: 1124.90 ± 108.99 mg/kg diet; 5000 mg/kg diet: 3548.67 ± 122.98 mg/kg diet. No cross-contamination of the control diet was detected and individual HBCD isomer levels detected in the diets are shown in Table S1.

3.1.2. General toxicity

The doses of T-HBCD administered in this study elicited minimal changes in food consumption; female Fischer rats had increased food consumption on day 21 in the 1250 mg/kg diet group and on days 14 and 21 in the 5000 mg/kg diet group (data not shown), and body weight was significantly higher at day 14 in the 1250 mg/kg diet group, and at days 14, 21 and at necropsy (final BW) in the 5000 mg/kg diet group (Table 1). This phenomenon was not evident in either the male Fischer rats or the other two strains tested.

At necropsy, no gross pathologies were noted for any of the test groups. Relative organ to body weight ratios (Table 2) were not significantly different in the Wistar strain; however, both Sprague Dawley (250 mg/kg diet, 4.36E-02 ± 0.14E-02; and 5000 mg/kg diet, 4.21E-02 ± 0.24E-02) and female Fischer (1250 mg/kg diet, 4.25E-02 ± 0.06E-02; and 5000 mg/kg diet, 4.35E-02 ± 0.16E-02) rats showed significantly higher liver weights to BW ratios relative to their corresponding controls (3.92E-02 ± 0.10E-02 and 3.86E-02 ± 0.16E-02, respectively); while male Fischer rats had significantly higher thyroid weights relative to BW ratios in the 1250 and 5000 mg/kg diet doses (10.9E-05 ± 0.9E-05 and 10.0E-05 ± 1.4E-05, respectively) than in the control diet (7.8 E − 05 ± 0.7E-05). Brain weight relative to BW ratios was significantly lower in female Fischer rats exposed to both the 1250 and 5000 mg/kg diet doses (9.0E-03 ± 0.2E-03 and 8.9E-03 ± 0.6E-03, respectively) than in the control diet (10.0E-03 ± 0.3E-03).

3.1.3. Haematology

Changes in haematological parameters are summarized in Table 3. Treatment with T-HBCD resulted in minimal changes in both Sprague Dawley and Wistar rats; however, both male and female Fischer rats showed significant changes in multiple haematological parameters following treatment at the two highest doses. Female Fischer rats had significantly increased RBC counts, HG concentration and HCT, while males showed increased MCH and MCHC at the highest dose. In males, WBC counts were significantly decreased, with fewer basophils, but with increased monocytes. Monocytes appear to be the most sensitive parameter in male Fischer rats, as all doses showed significant increases in their ratios relative to other WBCs.

3.1.4. Clinical chemistry

Exposure to T-HBCD resulted in altered clinical chemistry in all strains tested; however, Fischer rats were the most sensitive to the effects of T-HBCD based on number of altered parameters and doses at which they were altered (Table 4). Of the altered parameters, only two were changed in all strains and sexes: aspartate transaminase (AST) and creatine kinase (CK). In addition to AST and CK, several other parameters were significantly altered in Fischer rats, many of which point to thyroid (decreased T4, alkaline phosphatase (ALP) and AST, increased cholesterol and phosphorus) and pancreas (increased amylase and lipase) dysfunction.

Table 3
Changes in haematology parameters following exposure to T-HBCD.[1] $p \leq 0.05$ relative to control.

	Females							
T-HBCD added to diet (mg T-HBCD/kg diet)	Fischer				Sprague Dawley			
	0	250	1250	5000	0	250	1250	5000
White blood cells (10^9/L)	3.26 ± 0.73	3.04 ± 1.27	3.92 ± 0.50	3.08 ± 0.97	5.56 ± 1.83	6.22 ± 1.91	4.60 ± 1.64	7.18 ± 2.18
Red blood cells (10^{12}/L)	7.47 ± 0.30	7.35 ± 0.69	7.89 ± 0.12	**8.20 ± 0.11** [1]	7.12 ± 0.28	6.63 ± 0.26	6.90 ± 0.47	7.30 ± 0.24
Haemoglobin (g/L)	140.0 ± 6.86	145.0 ± 1.41	147.0 ± 1.87	**153.0 ± 3.24** [1]	142.0 ± 7.97	134.4 ± 5.41	142.2 ± 8.11	147.0 ± 2.55
Haematocrit (L/L)	0.388 ± 0.018	0.383 ± 0.038	0.411 ± 0.005	**0.429 ± 0.006** [1]	0.405 ± 0.025	0.378 ± 0.014	0.402 ± 0.021	0.423 ± 0.011
Mean corpuscular haemoglobin (pg)	18.74 ± 0.22	18.92 ± 0.26	18.64 ± 0.13	18.66 ± 0.29	19.92 ± 0.44	20.26 ± 0.30	20.64 ± 0.47	20.12 ± 0.37
Mean corpuscular haemoglobin concentration (g/L)	361.40 ± 1.14	364.00 ± 2.45	357.80 ± 1.30	356.80 ± 5.36	350.80 ± 2.39	355.60 ± 3.58	354.20 ± 3.56	347.40 ± 3.85
Neutrophils (10^9/L)	0.13 ± 0.02	0.14 ± 0.05	0.26 ± 0.06	0.20 ± 0.12	0.42 ± 0.18	0.34 ± 0.16	0.37 ± 0.08	0.65 ± 0.10
Neutrophils (Ratio)	4.18 ± 0.75	4.90 ± 1.21	**6.64 ± 1.07** [1]	6.26 ± 1.72	7.46 ± 1.96	5.48 ± 2.06	8.46 ± 1.89	8.28 ± 2.40
Lymphocytes (10^9/L)	3.04 ± 0.71	2.86 ± 1.21	3.57 ± 0.48	2.80 ± 0.82	4.98 ± 1.66	5.90 ± 1.81	4.09 ± 1.51	7.63 ± 3.70
Lymphocytes (Ratio)	94.30 ± 1.03	93.78 ± 1.25	**91.04 ± 1.45** [1]	91.80 ± 2.41	89.86 ± 2.10	92.08 ± 2.68	88.78 ± 1.81	86.86 ± 4.01
Monocytes (10^9/L)	0.032 ± 0.013	0.028 ± 0.019	0.066 ± 0.020	0.050 ± 0.041	0.108 ± 0.047	0.124 ± 0.063	0.110 ± 0.069	**0.272 ± 0.075** [1]
Monocytes (Ratio)	1.08 ± 0.43	0.90 ± 0.26	1.68 ± 0.38	1.42 ± 0.81	1.96 ± 0.43	1.94 ± 0.67	2.24 ± 0.61	3.44 ± 1.36
Basophils (10^9/L)	0.012 ± 0.005	0.008 ± 0.008	0.014 ± 0.006	0.014 ± 0.006	0.022 ± 0.013	0.026 ± 0.011	0.029 ± 0.046	0.050 ± 0.032
Basophils (Ratio)	0.32 ± 0.08	0.30 ± 0.10	0.38 ± 0.08	0.36 ± 0.06	0.38 ± 0.11	0.38 ± 0.08	0.28 ± 0.13	0.54 ± 0.11
	Males							
T-HBCD added to diet (mg T-HBCD/kg diet)	Wistar				Fischer			
	0	250	1250	5000	0	250	1250	5000
White blood cells (10^9/L)	3.98 ± 1.54	3.90 ± 1.23	3.82 ± 0.64	4.60 ± 0.83	7.02 ± 0.75	7.50 ± 0.42	7.76 ± 0.45	**5.14 ± 0.83** [1]
Red blood cells (10^{12}/L)	6.96 ± 0.21	7.36 ± 0.52	6.96 ± 0.36	7.19 ± 0.32	8.52 ± 0.14	8.64 ± 0.20	8.57 ± 0.13	8.25 ± 0.26
Haemoglobin (g/L)	135.0 ± 3.32	142.4 ± 5.90	140.2 ± 5.07	140.6 ± 4.34	150.8 ± 2.39	154.2 ± 2.28	153.6 ± 1.52	150.8 ± 3.70
Haematocrit (L/L)	0.389 ± 0.010	0.410 ± 0.018	0.399 ± 0.014	0.406 ± 0.013	0.447 ± 0.007	0.456 ± 0.008	0.453 ± 0.005	0.440 ± 0.012
Mean corpuscular haemoglobin (pg)	19.38 ± 0.68	19.38 ± 0.83	20.14 ± 0.48	19.56 ± 0.34	17.70 ± 0.07	17.88 ± 0.15	17.94 ± 0.06	**18.22 ± 0.26** [1]
Mean corpuscular haemoglobin concentration (g/L)	346.20 ± 3.03	347.60 ± 1.52	**350.60 ± 0.89** [1]	346.60 ± 1.52	337.00 ± 0.71	338.60 ± 1.52	338.80 ± 0.45	**342.00 ± 2.83** [1]
Neutrophils (10^9/L)	0.56 ± 0.30	0.51 ± 0.12	0.46 ± 0.09	0.53 ± 0.06	0.64 ± 0.29	0.69 ± 0.18	0.72 ± 0.12	0.44 ± 0.13
Neutrophils (Ratio)	14.60 ± 4.69	13.58 ± 2.84	12.12 ± 2.55	11.72 ± 2.34	9.10 ± 4.01	9.16 ± 2.32	9.34 ± 1.32	8.54 ± 1.62
Lymphocytes (10^9/L)	3.26 ± 1.36	3.23 ± 1.14	3.23 ± 0.61	3.82 ± 0.79	6.12 ± 0.64	6.47 ± 0.43	6.63 ± 0.34	**4.43 ± 0.68** [1]
Lymphocytes (Ratio)	81.80 ± 5.94	82.26 ± 3.83	83.82 ± 2.46	82.54 ± 3.76	87.14 ± 4.34	86.12 ± 2.69	85.74 ± 1.59	87.06 ± 2.38
Monocytes (10^9/L)	0.070 ± 0.039	0.108 ± 0.056	0.116 ± 0.031	**0.186 ± 0.027** [1]	0.192 ± 0.053	**0.272 ± 0.028** [1]	**0.270 ± 0.026** [1]	0.180 ± 0.043
Monocytes (Ratio)	1.84 ± 0.74	2.72 ± 1.07	3.08 ± 0.86	**4.12 ± 0.83** [1]	2.70 ± 0.55	**3.62 ± 0.34** [1]	**3.46 ± 0.13** [1]	**3.50 ± 0.47** [1]
Basophils (10^9/L)	0.014 ± 0.006	0.024 ± 0.009	0.016 ± 0.009	0.016 ± 0.009	0.044 ± 0.009	0.038 ± 0.005	0.038 ± 0.016	**0.024 ± 0.006** [1]
Basophils (Ratio)	0.30 ± 0.10	**0.60 ± 0.10** [1]	0.42 ± 0.13	0.38 ± 0.08	0.62 ± 0.08	0.48 ± 0.05	0.48 ± 0.16	0.48 ± 0.08

Table 4
Changes in clinical chemistry parameters following exposure to T-HBCD.[1] $p \leq 0.05$ relative to control.

	Females							
T-HBCD added to diet (mg T-HBCD/kg diet)	Fischer				Sprague Dawley			
	0	250	1250	5000	0	250	1250	5000
A/G Ratio	2.58 ± 0.05	2.40 ± 0.12	2.28 ± 0.21	2.40 ± 0.19	3.50 ± 0.61	2.94 ± 0.21	2.90 ± 0.19	**2.78 ± 0.22** [1]
Albumin (g/L)	45.80 ± 2.17	44.40 ± 2.30	47.40 ± 2.07	46.40 ± 1.67	50.00 ± 2.45	47.20 ± 3.03	51.40 ± 2.70	50.00 ± 2.74
Alkaline Phosphatase (U/L)	223.8 ± 20.7	**157.8 ± 7.7** [1]	**146.4 ± 10.3** [1]	**147.8 ± 22.6** [1]	142.6 ± 32.6	151.8 ± 37.4	113.0 ± 15.2	120.6 ± 42.8
Amylase (U/L)	1929.0 ± 113.2	1943.2 ± 150.6	**2260.2 ± 120.5** [1]	**2196.2 ± 115.0** [1]	2032.2 ± 94.8	2113.8 ± 132.6	**2359.0 ± 128.4** [1]	2320.0 ± 247.9
Aspartate Transaminase (U/L)	87.40 ± 14.94	65.60 ± 6.62	57.00 ± 3.81	**53.00 ± 3.94** [1]	118.4 ± 23.5	108.20 ± 21.3	**80.2 ± 13.1** [1]	**68.6 ± 20.1** [1]
Bicarbonate (mmol/L)	27.2 ± 2.17	27.60 ± 3.85	26.80 ± 4.21	30.20 ± 3.11	27.40 ± 2.40	30.00 ± 1.41	29.20 ± 0.84	28.20 ± 1.48
Bilirubin (Total) (umol/L)	1.80 ± 0.30	1.64 ± 0.38	1.16 ± 0.39	1.48 ± 0.79	2.42 ± 0.79	1.48 ± 0.72	2.10 ± 0.68	1.50 ± 0.89
Calcium (mmol/L)	2.79 ± 0.10	2.84 ± 0.09	2.93 ± 0.11	2.89 ± 0.09	2.69 ± 0.09	2.77 ± 0.04	**2.82 ± 0.10** [1]	**2.86 ± 0.04** [1]
Chloride (mmol/L)	102.2 ± 4.0	99.6 ± 0.6	96.6 ± 3.7	**94.2 ± 3.1** [1]	102.4 ± 1.5	101.8 ± 1.5	103.4 ± 1.3	104.0 ± 1.6
Cholesterol (mmol/L)	2.62 ± 0.18	2.80 ± 0.25	**3.30 ± 0.19** [1]	**3.34 ± 0.15** [1]	1.92 ± 0.38	2.40 ± 0.20	**3.00 ± 0.62** [1]	**2.92 ± 0.42** [1]
Creatine Kinase (U/L)	695.6 ± 195.3	**424.6 ± 80.7** [1]	**283.2 ± 70.5** [1]	**249.0 ± 34.7** [1]	864.6 ± 312.9	857.2 ± 293.1	418.6 ± 219.8	**359.8 ± 193.1** [1]
Creatinine (umol/L)	30.40 ± 3.05	29.00 ± 1.41	27.20 ± 2.59	**23.40 ± 2.70** [1]	33.80 ± 3.56	31.0 ± 1.87	30.40 ± 1.67	29.80 ± 3.56
Globulin (g/L)	17.80 ± 0.84	18.60 ± 1.14	**21.00 ± 1.00** [1]	**19.60 ± 0.89** [1]	14.60 ± 1.95	16.20 ± 1.48	17.80 ± 2.05	**18.00 ± 1.00** [1]
Glucose (mmol/L)	10.08 ± 0.58	**12.14 ± 1.57** [1]	11.00 ± 1.05	11.86 ± 0.97	10.06 ± 0.51	11.12 ± 2.27	11.42 ± 0.99	11.44 ± 1.07
Lipase (U/L)	9.20 ± 1.48	16.40 ± 7.67	16.40 ± 4.51	**18.60 ± 2.61** [1]	8.00 ± 3.08	16.20 ± 9.47	17.60 ± 3.91	12.40 ± 3.58
Magnesium (mmol/L)	1.07 ± 0.07	1.07 ± 0.10	0.98 ± 0.04	0.97 ± 0.09	1.14 ± 0.05	1.09 ± 0.05	1.04 ± 0.07	**0.96 ± 0.09** [1]
Phosphorous (mmol/L)	2.50 ± 0.20	**3.08 ± 0.25** [1]	**2.88 ± 0.13** [1]	**2.94 ± 0.09** [1]	2.50 ± 0.24	2.78 ± 0.13	2.68 ± 0.23	2.66 ± 0.09
Sodium (mmol/L)	146.4 ± 5.1	141.6 ± 1.8	138.6 ± 5.3	**135.8 ± 4.8** [1]	142.6 ± 2.1	142.4 ± 0.6	141.8 ± 1.5	142.0 ± 0.71
Total Protein (g/L)	63.60 ± 2.97	63.00 ± 3.16	68.60 ± 2.19	66.20 ± 2.49	64.60 ± 1.52	63.40 ± 4.16	69.20 ± 4.71	68.00 ± 2.92
Triglycerides (mmol/L)	1.56 ± 0.55	1.56 ± 0.39	**2.39 ± 0.44** [1]	**2.47 ± 0.54** [1]	1.40 ± 0.32	1.94 ± 0.49	1.60 ± 0.36	1.89 ± 0.94
Total T4 (nmol/L)	64.54 ± 8.42	60.20 ± 11.51	54.68 ± 12.02	46.96 ± 4.80	35.36 ± 13.08	43.32 ± 7.91	45.62 ± 5.59	53.54 ± 14.83
	Males							
T-HBCD added to diet (mg T-HBCD/kg diet)	Wistar				Fischer			
	0	250	1250	5000	0	250	1250	5000
A/G Ratio	3.34 ± 0.38	3.32 ± 0.21	3.12 ± 0.34	2.90 ± 0.30	2.10 ± 0.10	2.00 ± 0.07	**1.96 ± 0.06** [1]	2.02 ± 0.05
Albumin (g/L)	42.20 ± 2.39	45.20 ± 3.42	43.60 ± 2.07	45.60 ± 3.78	41.20 ± 1.10	41.60 ± 0.55	41.20 ± 0.84	**42.80 ± 0.45** [1]
Alkaline Phosphatase (U/L)	183.4 ± 42.7	118.4 ± 29.8	**90.6 ± 33.3** [1]	126.2 ± 46.7	220.2 ± 9.0	205.2 ± 19.4	**191.8 ± 9.8** [1]	**182.2 ± 8.5** [1]
Amylase (U/L)	1567.6 ± 278.9	1867.2 ± 152.0	2027.8 ± 389.7	1837.2 ± 368.5	2839.2 ± 73.1	2959.0 ± 68.3	2959.0 ± 136.1	**3068.8 ± 96.3** [1]
Aspartate Transaminase (U/L)	107.0 ± 18.6	96.80 ± 5.5	**72.6 ± 15.3** [1]	**64.4 ± 6.1** [1]	86.80 ± 10.04	77.40 ± 4.10	**68.00 ± 4.85** [1]	**61.00 ± 5.96** [1]
Bicarbonate (mmol/L)	26.80 ± 2.59	27.20 ± 1.48	28.00 ± 1.87	28.60 ± 1.67	32.20 ± 1.48	30.60 ± 1.52	28.80 ± 1.79	**29.00 ± 1.00** [1]
Bilirubin (Total) (umol/L)	2.72 ± 0.13	2.36 ± 0.23	**1.80 ± 0.61** [1]	1.90 ± 0.62	1.68 ± 0.48	1.54 ± 0.15	1.40 ± 0.58	1.08 ± 0.41
Calcium (mmol/L)	2.42 ± 0.06	2.53 ± 0.09	2.47 ± 0.10	**2.62 ± 0.11** [1]	2.84 ± 0.09	2.94 ± 0.07	2.90 ± 0.08	**3.02 ± 0.04** [1]
Chloride (mmol/L)	99.4 ± 2.3	98.8 ± 3.0	99.4 ± 1.3	101.2 ± 1.3	97.4 ± 2.1	100.4 ± 2.3	100.0 ± 1.2	**102.4 ± 0.6** [1]
Cholesterol (mmol/L)	1.74 ± 0.29	1.78 ± 0.27	2.16 ± 0.36	2.24 ± 0.40	2.42 ± 0.16	2.70 ± 0.16	2.46 ± 0.11	2.62 ± 0.27
Creatine Kinase (U/L)	960.0 ± 270.1	722.6 ± 194.6	557.0 ± 276.7	**353.0 ± 142.4** [1]	799.4 ± 191.6	678.0 ± 75.2	**429.6 ± 91.8** [1]	**229.6 ± 60.8** [1]
Creatinine (umol/L)	24.20 ± 3.35	24.40 ± 2.19	**19.20 ± 2.78** [1]	20.80 ± 1.92	23.80 ± 2.17	22.40 ± 2.07	21.40 ± 2.30	21.00 ± 1.23
Globulin (g/L)	12.80 ± 1.92	13.60 ± 1.52	14.20 ± 1.79	15.60 ± 2.07	20.00 ± 1.41	21.00 ± 0.71	21.20 ± 0.45	21.60 ± 0.55
Glucose (mmol/L)	9.96 ± 1.27	8.70 ± 0.96	8.48 ± 0.54	9.66 ± 0.49	9.92 ± 1.87	9.38 ± 0.73	9.12 ± 0.34	11.96 ± 0.47
Lipase (U/L)	7.20 ± 1.10	6.00 ± 1.58	7.40 ± 2.07	**11.20 ± 2.78** [1]	14.80 ± 3.35	13.60 ± 1.52	13.60 ± 1.82	17.20 ± 3.77
Magnesium (mmol/L)	1.11 ± 0.07	1.01 ± 0.09	**0.93 ± 0.06** [1]	**0.91 ± 0.07** [1]	0.89 ± 0.04	0.85 ± 0.05	**0.79 ± 0.05** [1]	**0.80 ± 0.04** [1]
Phosphorous (mmol/L)	2.40 ± 0.22	2.58 ± 0.28	2.50 ± 0.35	2.54 ± 0.13	2.84 ± 0.11	3.26 ± 0.15	3.20 ± 0.26	**3.42 ± 0.13** [1]

(*continued on next page*)

Table 4 (*continued*)

	Males									
T-HBCD added to diet (mg T-HBCD/kg diet)	Wistar					Fischer				
	0	250	1250	5000		0	250	1250	5000	
Sodium (mmol/L)	139.4 ± 3.8	138.8 ± 3.6	135.0 ± 2.4	137.4 ± 1.5		144.0 ± 2.4	146.2 ± 3.6	143.2 ± 2.3	144.2 ± 0.5	
Total Protein (g/L)	55.20 ± 4.38	58.60 ± 4.67	57.80 ± 3.27	60.40 ± 4.56		61.20 ± 2.28	62.60 ± 0.89	62.40 ± 0.89	**64.40 ± 0.89** [1]	
Triglycerides (mmol/L)	1.32 ± 0.34	0.93 ± 0.27	1.38 ± 0.28	1.91 ± 0.89		2.11 ± 0.60	2.31 ± 0.52	2.24 ± 0.39	2.43 ± 0.32	
Total T4 (nmol/L)	58.26 ± 10.05	**28.22 ± 6.26** [1]	**38.00 ± 8.93** [1]	45.34 ± 10.86		98.50 ± 12.43	98.72 ± 3.64	87.36 ± 5.10	**81.34 ± 5.39** [1]	

3.1.5. Residue analysis

T-HBCD concentrations were measured and expressed as the proportion of α- or γ-HBCD per gram of lipid in the liver, serum and adipose tissues. α and γ isomer tissue concentrations are presented in Table 5. The β isomer was not detected in any tissues tested for residue (serum, liver or adipose). As expected, the γ isomer was more abundant than the α isomer in all tissues. Values of either isomer were highest in the serum and adipose tissue, with liver having the lowest concentrations. Male and female Fischer rats had significantly different patterns of HBCD accumulation, with both isomers accumulating in females in higher concentration compared to that of the males, with the exception of the α isomer in serum.

3.1.6. Histopathological analysis

Liver, kidney, thyroid, spleen, thymus, adrenals, and heart were all examined for histopathologic abnormalities in all animals in this study. Those organs which exhibited treatment-related pathology are discussed below. All other organs displayed no significant lesions attributable to treatment and will not be discussed. A summary of the incidence and severity can be found in Table S2 in Supplemental Information.

3.1.6.1. Liver. No significant lesions were found in the liver of any animals. Common background changes (minimal to mild) were seen in all groups. These changes include multifocal necrosis, random sinusoidal granulomas, hepatocellular glycogen accumulation, and fatty acid change or vacuolation of hepatocytes.

3.1.6.2. Kidney. Mild chronic nephropathy and mild multifocal tubular mineralization was present in all males in all treatment groups. Mild focal nephropathy was seen in occasional females in all dose groups and controls.

3.1.6.3. Thyroid. In the thyroid of exposed animals, there was evidence of mild follicular hypertrophy. A summary of thyroid lesions can be found in Table S2. Follicular cell hypertrophy was significantly increased in all strains and both sexes at the two highest doses. Specifically, in female Fischer rats, mild follicular hypertrophy was noted in all animals from both the 1250 and 5000 mg/kg diet groups, Wistar rats showed minimal to mild hypertrophy in the 1250 and 5000 mg/kg diet groups. In Sprague Dawley rats, two animals showed minimal evidence of hypertrophy in the 1250 mg/kg diet group; however, all animals in the 5000 mg/kg diet group exhibited mild follicular cell hypertrophy. In male Fischer rats, three of the five animals from the 1250 mg/kg diet group showed minimal to mild hypertrophy and four of the five animals from the 5000 mg/kg diet group displayed follicular hypertrophy. There was a significant increase in colloid depletion in a dose-dependent manner in male rats. There was no evidence of colloid depletion in female treated rats, with the exception of one high dose Fischer female. Mild to moderate colloid vacuolation was also evident and increased in HBCD-fed male Fischers; however, vacuolation was more evident in all female dose groups, irrespective of strain (Fig. 1).

3.1.6.4. Spleen. A summary of splenic lesions can be found in Table S2. Without exception, no lesions were identified in any dose group or strain in any of the females tested. In males, T-cell (periarteriolar lymphoid sheaths; PALS) and B-cell (follicles) areas and marginal zone (MZ) area were significantly reduced in two of the five animals from the 1250 mg/kg diet group. In the 5000 mg/kg diet group, four of five spleens had significant atrophy of moderate severity in both T- and B-dependent areas (Fig. 2).

3.1.6.5. Thymus. Thymus samples were not available for all females. With the exception of one male in the 1250 mg/kg diet group whose thymus exhibited mildly increased cortical macrophages, all male thymus sections from all treatment groups were within normal limits.

Table 5
α and γ HBCD congener tissue concentration (ug/g) for Fischer, Sprague Dawley and Wistar rats following exposure to T- HBCD.[1] $p \leq 0.05$ relative to control;[2] $p \leq 0.05$ relative to 250 mg T-HBCD/kg diet;[3] $p \leq 0.05$ relative to 1250 mg T-HBCD/kg diet. ND = not detected, limit of detection for α and γ in each tissue: serum 0.01 μg/g, liver 0.01 μg/g, adipose 0.02 μg/g.

	HBCD added to diet (mg T-HBCD/kg diet)	Females											
		Fischer				Sprague Dawley				Wistar			
		0	250	1250	5000	0	250	1250	5000	0	250	1250	5000
Alpha	Adipose	ND	9.38 ± 1.05	**23.24 ± 3.10** [1]	**65.19 ± 4.73** [1,2]	0.02 ± 0.04	11.09 ± 2.66	**25.97 ± 3.03** [1]	**72.65 ± 9.19** [1,2]	ND	**10.98 ± 3.00** [1]	**29.52 ± 5.99** [1]	**73.42 ± 8.68** [1]
	Liver	ND	ND	11.15 ± 1.22	**41.07 ± 6.12** [1,2]	ND	ND	9.35 ± 2.12	**38.22 ± 12.70** [1,2]	ND	ND	10.86 ± 6.53	**39.61 ± 6.31** [1,2]
	Serum	ND	ND	29.32 ± 0.79	**69.25 ± 8.92** [1]	ND	ND	17.86 ± 10.23	**78.98 ± 13.85** [1,2]	ND	ND	27.32 ± 9.56	**67.62 ± 7.54** [1,2]
Gamma	Adipose	ND	219.91 ± 10.49	**546.75 ± 69.18** [1]	**554.26 ± 26.12** [1]	0.07 ± 0.15	**267.67 ± 41.62** [1]	**611.86 ± 56.17** [1]	**590.37 ± 47.86** [1]	ND	**264.65 ± 72.47** [1]	**675.26 ± 97.47** [1]	**660.58 ± 66.24** [1]
	Liver	ND	181.34 ± 23.83	**249.14 ± 33.08** [1]	**251.17 ± 76.58** [1]	ND	**257.61 ± 72.70** [1]	**212.37 ± 37.71** [1]	**233.50 ± 66.69** [1]	ND	177.65 ± 32.97	**269.09 ± 114.45** [1]	**277.43 ± 14.28** [1]
	Serum	0.42 ± 0.93	**562.34 ± 151.98** [1]	**799.27 ± 153.61** [1]	**702.32 ± 116.00** [1]	ND	706.86 ± 83.76	**852.02 ± 285.88** [1]	741.62 ± 129.94	ND	630.80 ± 83.83	**844.48 ± 309.08** [1]	**777.14 ± 97.20** [1]

	HBCD added to diet (mg T-HBCD/kg diet)	Males			
		Fischer			
		0	250	1250	5000
Alpha	Adipose	0.55 ± 0.26	1.43 ± 3.20	**20.19 ± 9.31** [1,2]	**47.11 ± 4.09** [1,2,3]
	Liver	0.83 ± 1.18	ND	2.83 ± 2.19	**20.46 ± 4.10** [1,2,3]
	Serum	ND	ND	**31.76 ± 5.43** [1,2]	**70.10 ± 7.00** [1,2,3]
Gamma	Adipose	0.05 ± .02	**129.48 ± 10.32** [1]	**382.88 ± 35.27** [1,2]	**420.57 ± 17.34** [1,2,3]
	Liver	0.04 ± 0.07	**79.38 ± 18.97** [1]	**157.33 ± 47.99** [1,2]	**138.40 ± 22.55** [1,2]
	Serum	ND	**294.88 ± 29.77** [1]	**672.27 ± 191.74** [1,2]	**536.78 ± 61.15** [1,2]

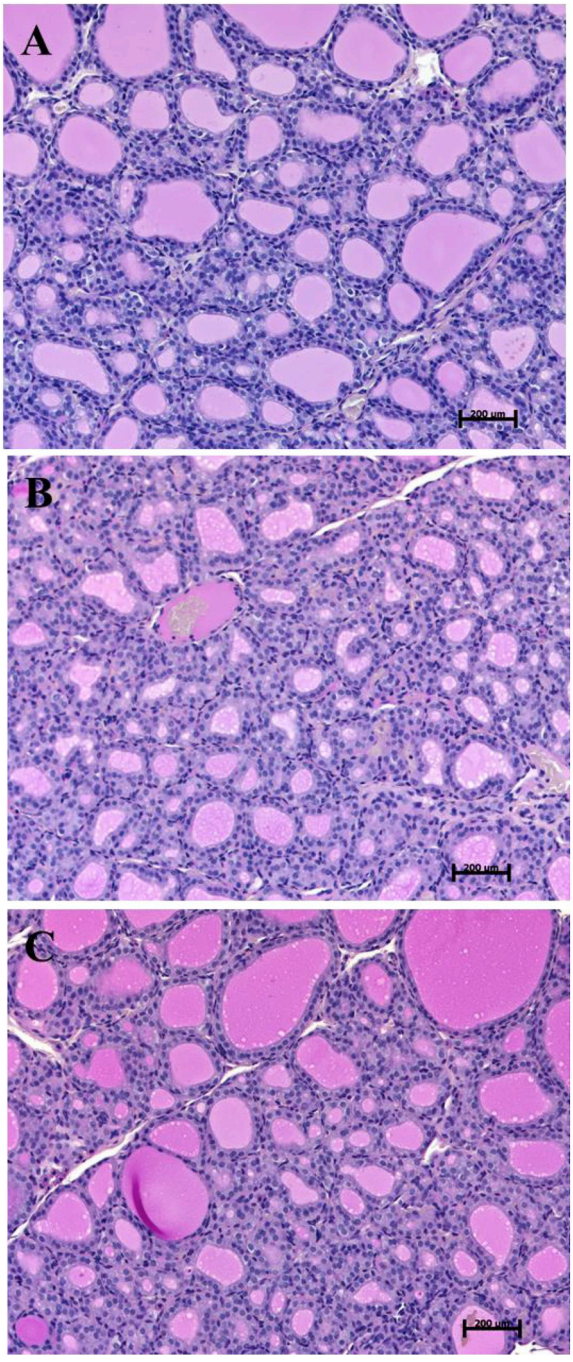

Fig. 1. Thyroid lesions in T-HBCD-treated male Fischer rats. A) Control, B) 1250 mg/kg diet, C) 5000 mg/kg diet animals. Magnification 20×.

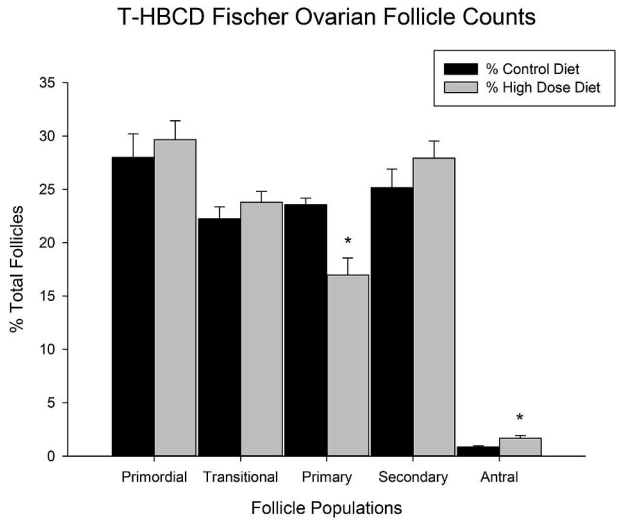

Fig. 3. T-HBCD treatment alters ovarian follicle distribution in Fischer rats. Ovaries from high dose and control Fischer rats were examined and the number of follicles at each stage of development was determined. Ovaries from rats fed a 5000 mg/kg T-HBCD diet had a significantly lower percentage of primary follicles and a greater percentage of antral follicles in the high dose group compared to control. *p < 0.05.

3.1.6.6. Heart. Mild myocardial fiber degeneration (lipid type) was noted in two female Wistar rats in the 250 mg/kg diet group. Acute mild multifocal myofiber necrosis was a common lesion in all other dosed rats and controls.

3.1.7. T-HBCD depletes primary ovarian follicles in Fischer rats

Evaluation of ovaries from Fischer rats exposed to T-HBCD revealed a significant reduction in the number of growing follicles, specifically the primary stage of development ($p = 0.003$) compared to control animals. Total number of follicles and follicles at other stages of development were not significantly different between treatment groups; however, when normalized to the total number of follicles, there was a significantly lower percentage of primary follicles (16.97 ± 1.59% vs 23.57 ± 0.61%; $p = 0.005$) and a greater percentage of antral follicles (1.67 ± 0.25% vs 0.87 ± 0.10%; $p = 0.02$) in the high dose group compared to control (Fig. 3).

3.1.8. T-HBCD alters immunotoxicity parameters

No change in splenocyte proliferation was observed in either Sprague Dawley or Wistar rats (data not shown). Significant decreases in splenocyte proliferation were found in both female and male Fischer rats (Table S4). Female Fischers had a decrease in splenocyte proliferation in all populations irrespective of challenge, male Fischers had decreased proliferation in LPS-stimulated cultures at the highest dose tested. However, when normalized to unstimulated controls, the

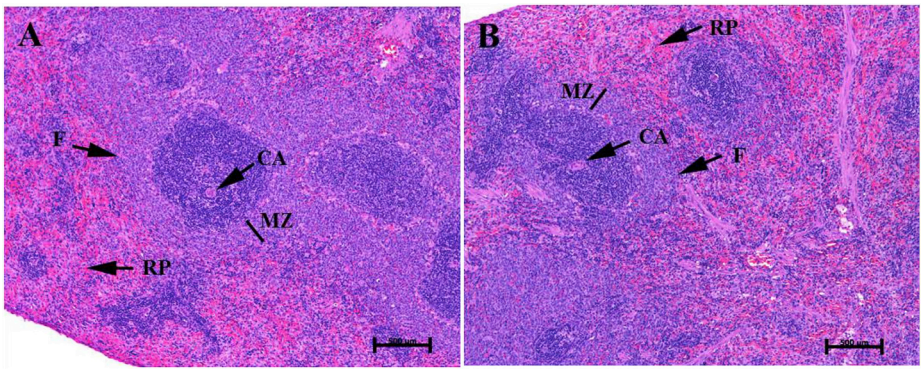

Fig. 2. Splenic lesions in T-HBCD treated male Fischer rats vs control. A) Control, B) 5000 mg/kg diet dose. Mild to moderate atrophy of the T-cell and B-cell regions is apparent in treated tissues. CA = central artery; F = follicle; MZ = marginal zone; RP = red pulp. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Benchmark doses and benchmark dose lower confidence limits for selected endpoints in Fischer, Sprague Dawley and Wistar rats exposed to T-HBCD in diet for 28 days.

Parameter[a]	BMD (range of BMDs for all acceptable models) in units of mg/kg bw/d	BMDL (range of BMDLs for all acceptable models) in units of mg/kg bw/d[b]
Fischer Females		
Increased BW (day 14)	244.48 (244.48–508.72)	21.30 (21.30–342.02)
Increased BW (day 21)	213.94	45.79
Increased BW (final)	192.42 (192.42–443.55)	21.33 (21.33–305.04)
Increased relative liver weight (/BW)	99.55	47.66
Decreased serum AST (U/L)	9.58	4.50
Increased serum cholesterol (mmol/L)	13.67	7.50
Decreased serum CK (U/L)	9.54	4.65
Decreased serum creatinine (mmol/L)	143.04 (66.25–158.05)	96.69 (19.54–110.57)
Decreased serum sodium (mmol/L)	26.05 (18.10–26.05)	5.58 (3.27–5.58)
Increased serum triglycerides (mmol/L)	37.80	14.60
Decreased blood lymphocytes (ratio)	27.87	10.21
Increased blood neutrophils (ratio)	24.11	8.40
Fischer Males		
Increased serum albumin (g/L)	314.29 (195.02–376.83)	141.01 (97.52–227.50)
Decreased serum ALP (U/L)	13.74 (13.74–182.56)	4.40 (4.40–122.08)
Increased serum amylase (U/L)	219.41 (219.41–223.41)	139.96 (139.96–144.32)
Decreased serum AST (U/L)	11.39 (11.39–17.73)	4.68 (4.68–7.13)
Decreased serum bicarbonate (mmol/L)	12.68	4.95
Increased serum chloride (mmol/L)	270.91 (175.39–270.91)	182.66
Decreased serum CK (U/L)	18.83 (18.83–24.35)	14.45
Decreased serum magnesium (mmol/L)	16.48	6.15
Increased serum total protein (g/L)	215.11 (116.03–217.61)	142.70 (48.47–145.36)
Decreased blood WBC (10^9/L)	260.38	186.66
Decreased blood lymphocytes (10^9/L)	256.02 (206.47–256.02)	159.18 (142.24–159.18)
Decreased blood $CD3^+$ cells (10^9/L)	355.40 (196.84–368.48)	137.01 (137.01–225.49)
Decreased blood CD3 + 4 + cells (10^9/L)	349.27 (191.47–362.08)	131.09 (129.94–165.78)
Decreased blood CD3 + 8 + cells (10^9/L)	371.35 (216.69–371.35)	156.82 (152.99–185.18)
Increased blood CD161a + cells (% lymphocytes)	102.18 (102.12–129.53)	31.54 (31.54–85.62)
Increased incidence of thyroid follicular cell hypertrophy	20.02 (12.78–64.10)	10.55 (5.01–35.43)
Increased incidence of thyroid colloid depletion	15.63 (15.63–86.72)	7.57 (7.57–29.16)
Increased incidence of decreased cell density in spleen PALS	32.58 (32.58–118.37)	16.04 (6.75–57.07)
Increased incidence of decreased area in spleen PALS	25.50 (25.50–84.99)	13.08 (4.61–44.01)
Increased incidence of decreased area in spleen marginal zone	36.94 (23.34–110.29)	18.60 (7.82–58.28)
Sprague Dawley Females		
Increased serum amylase (U/L)	25.88	9.43
Decreased serum AST (U/L)	33.25 (33.25–146.97)	12.58 (12.58–87.86)
Increased serum cholesterol (mmol/L)	14.39	5.93
Decreased serum CK (U/L)	39.28 (39.28–129.98)	15.14 (15.14–53.35)
Increased serum globulin (g/L)	19.24	6.03
Decreased serum magnesium (mmol/L)	167.83 (49.19–167.83)	114.97 (11.59–114.97)
Increased blood monocyte ratio	155.47 (129.44–165.59)	96.64 (70.90–96.67)
Increased blood monocytes (10^9/L)	385.36 (151.25–385.36)	121.73 (105.74–209.47)
Decreased blood $CD3^+$ cells (% lymphocytes)	20.69	8.28
Increased blood CD45RA + cells (% lymphocytes)	22.46	8.70
Decreased blood T cell/B cell ratio	13.58	5.79
Wistar Females		
Decreased serum CK (U/L)	24.26 (24.26–221.75)	5.30 (5.30–146.87)
Increased serum lipase (U/L)	214.59 (183.50–255.68)	160.16 (76.35–161.04)
Decreased serum magnesium (mmol/L)	12.11	4.93
Decreased serum total bilirubin (umol/L)	5.27	2.45
Increased blood monocyte ratio	214.56 (72.62–258.31)	143.26 (11.76–188.83)
Increased blood monocytes (10^9/L)	109.97 (105.41–225.22)	38.67 (23.21–172.09)

[a] Abbreviations: AST, aspartate transaminase; ALP, alkaline phosphatase; BMD, benchmark dose; BMDL, benchmark dose 95% lower confidence limit; BMR, benchmark response; BW, body weight; CK, creatine kinase; PALS, periarteriolar lymphoid sheath; WBC, white blood cells. Only results from data amenable to modelling by BMD software, as described in the Methods and Materials section, are included.

[b] All data generated in the study were modelled using US EPA's BMD software, version 2.5, as described in the Methods section. BMD and BMDL values are for the model of best fit according to criteria described in the Methods section; ranges are for all models with acceptable fit. Final BW, organ weight, and lesion incidence data were analysed using a default benchmark response (BMR) of 10% change in the mean, yielding a $BMDL_{10}$. For all other data the default BMR of one control standard deviation (SD) of the control mean was applied to the modelled endpoints, producing a $BMDL_{1SD}$. The lower limit of a one-sided 95% confidence interval localized on each parameter was used to derive the BMDLs in this table (EPA, 2012).

treatment effect was not evident in female Fischers despite HBCD appearing to have a generalized effect on splenocyte viability (proliferation was decreased in unstimulated as well as LPS- and ConA-stimulated cultures at multiple doses). Contrary to female Fischers, however, male Fischers showed a treatment effect on splenocyte proliferation following LPS challenge at all doses. In addition, female Fischers showed a decrease in IgG serum immunoglobulin at all doses although no other strain or male Fischers showed similar effects (data not shown). Finally,

Table 7
Mean daily A-HBCD consumption, initial body weights, final body weights for female and male Fischer rats, for 28 days.[a] calculated from mean weekly food consumption/mean BW for each dose group over entire 28 day exposure period;[1] $p \leq 0.05$ relative to control.

HBCD added to diet (mg A-HBCD/kg diet)	Female Fischer				Male Fischer			
	0	250	1250	5000	0	250	1250	5000
Calculated daily A-HBCD consumption[a] (mg/kg BW/day)	0.00 ± 0.00	19.46 ± 3.94	99.02 ± 16.56	362.53 ± 43.37	0.00 ± 0.00	19.16 ± 3.45	97.30 ± 16.57	395.30 ± 35.16
Initial BW (g)	123.84 ± 6.59	124.92 ± 4.81	124.04 ± 3.35	124.60 ± 4.90	142.72 ± 7.18	140.52 ± 11.35	137.16 ± 10.72	133.44 ± 11.66
Day 7 BW (g)	142.15 ± 7.40	141.96 ± 3.22	145.12 ± 4.29	144.52 ± 3.93	180.08 ± 9.87	177.36 ± 12.61	176.04 ± 12.60	169.76 ± 13.07
Day 14 BW (g)	153.85 ± 9.82	156.20 ± 3.81	162.84 ± 4.27	154.52 ± 4.95	211.28 ± 10.18	208.72 ± 13.81	210.28 ± 14.66	195.00 ± 10.94
Day 21 BW (g)	165.35 ± 10.99	168.12 ± 4.95	178.12 ± 4.20	166.28 ± 5.11	236.20 ± 10.34	235.32 ± 13.26	237.00 ± 15.61	220.76 ± 13.24
Final BW (g)	174.25 ± 12.50	176.48 ± 5.37	187.00 ± 3.93	172.04 ± 6.70	254.40 ± 8.92	254.96 ± 11.73	257.52 ± 18.00	243.64 ± 13.88

blood lymphocyte populations were unchanged in female Fischers and Wistars while in both Sprague Dawley and male Fischers treatment resulted in decreased T cell and increased B cell populations, leading to a decreased T cell/B cell ratio. In males specifically, both helper T (T_H) and cytotoxic T (T_C) cell populations were decreased and an increase in natural killer (NK) cells was apparent. No changes in thymus lymphocytes were evident in any treatment group (data not shown).

3.1.9. BMD modelling

Although all study endpoints were modelled, not all endpoints could adequately be fitted to the BMD models. For those that fit the available models, BMDs and corresponding BMDLs are summarized in Table 6. In female rats, the lowest BMDLs were decreased creatine kinase (Fischer BMDL 4.65 mg/kg BW/d; Sprague Dawley $BMDL_{10}$ 15.14 mg/kg BW/d; Wistar BMDL 5.30 mg/kg BW/d), increased cholesterol (Fischer 7.50 mg/kg BW/d; Sprague Dawley 5.93 mg/kg BW/d; Wistars were not able to be modelled) and decreased AST (Fischer 4.5 mg/kg BW/d; Sprague Dawley 12.58 mg/kg BW/d; Wistar rats were not able to be modelled). In male rats the lowest BMDLs were decreased serum ALP (4.40 mg/kg BW/d), decreased AST (4.68 mg/kg BW/d), decreased bicarbonate (4.95 mg/kg BW/d), decreased creatine kinase (14.45 mg/kg BW/d) and increased incidence of thyroid colloid depletion (7.57 mg/kg BW/d).

3.2. Alpha HBCD study

Based on the findings of our technical HBCD study with the three strains, we determined that Fischer rats were most sensitive to the effects of HBCD. Given that α-HBCD is preferentially bioaccumulated (Tang et al., 2015) and thus has the potential to elicit the greatest response to exposure, we endeavoured to examine the effects of an α-HBCD enriched diet on both male and female Fischer rats. The results of that study are presented below.

3.2.1. Exposure to the test compound αHBCD enriched diet

Analysis of the experimental diets indicated that the achieved levels in the experimental feeds of total HBCD in A-HBCD diets were as follows: Control: 0.00 ± 0.00 mg/kg diet; 250 mg/kg diet: 200.12 ± 21.14 mg/kg diet; 1250 mg/kg diet: 897.85 ± 57.28 mg/kg diet; 5000 mg/kg diet: 3938.21 ± 1054.80 mg/kg diet. No cross-contamination of the control diet was detected and individual HBCD isomer levels detected in the diets are shown in Table S1.

3.2.2. General toxicity

The doses of A-HBCD administered in this study elicited minimal changes in food consumption: females had increased food consumption on day 21 in both the 1250 mg/kg and 5000 mg/kg diet groups, while males had increased consumption on day 27 in the 5000 mg/kg diet group (data not shown); however, BWs were not different at any time during the study (Table 7).

At necropsy, no gross pathologies were noted for any of the test groups. Relative organ weight/BW ratios of the livers (Table 8) of both female (1250 mg/kg diet, 4.70E-02 ± 0.14E-02; and 5000 mg/kg diet, 6.42E-02 ± 0.12E-02) and male (1250 mg/kg diet, 4.76E-02 ± 0.18E-02; and 5000 mg/kg diet, 6.03E-02 ± 0.10E-02) rats showed significantly higher liver weights relative to their corresponding controls (3.88E-02 ± 0.10E-02and 4.16E-02 ± 0.22E-02, respectively), suggesting an adaptive mechanism to exposure. Thyroid weights were increased at the highest dose for both females and males (1.79E-04 ± 0.2E-04 and 01.93E-04 ± 0.4E-04, respectively). Both thymus and brain weights were decreased in females (in the 5000 mg/kg diet group (1.85E-03 ± 0.1E-03) and in the 1250 mg/kg diet group (1.01E-02 ± 0.01E-02), respectively) whereas male Fischers showed no differences.

3.2.3. Haematology

Changes in haematological parameters for those rats exposed to A-HBCD are summarized in Table 9. Male rats had elevated HG and MCH at the two highest doses while both sexes of Fischer rats had slightly, but significantly, elevated MCHC at the two highest doses. Neutrophils and monocytes were significantly elevated at the highest dose for both male and females, while lymphocytes were only depressed in the females at the highest dose.

3.2.4. Clinical chemistry

Exposure to A-HBCD resulted in several altered parameters of clinical chemistry in both female and male rats (Table 10). Similar to exposure to T-HBCD, A-HBCD elicited a significant reduction in CK levels at all doses administered. Total protein and chloride were increased in both females and males at the highest dose, respectively. Total T4 levels were significantly decreased in both sexes, pointing to possible thyroid dysfunction, which is also indicated by increased phosphorus and amylase levels. In both males and females, exposure to A-HBCD resulted in increases in both serum amylase and lipase.

3.2.5. Residue analysis

A-HBCD residue concentrations were measured and expressed as lipid adjusted concentrations in liver, serum and adipose tissues. Tissue concentrations are presented in Table 11. The α isomer was more abundant than the γ isomer in all tissues tested. Values of either isomer were highest in the serum and adipose tissue, with liver having the lowest concentrations. Females had significantly higher A-HBCD accumulation, with both isomers accumulating in females in higher concentrations relative to those of the males in all tissues and doses, with the exception of the α isomer in liver at the highest dose.

Table 8
Relative organs weights for female and male Fischer rats (organ wt/BW) exposed to A-HBCD.[1] $p \leq 0.05$ relative to control.

HBCD added to diet (mg A-HBCD/kg diet)	Females				Males			
	0	250	1250	5000	0	250	1250	5000
Liver	3.88E-02 ± 0.10E-02	3.97E-02 ± 0.10E-02	**4.70E-02 ± 0.14E-02** [1]	**6.42E-02 ± 0.12E-02** [1]	4.16E-02 ± 0.22E-02	4.36E-02 ± 0.09E-02	**4.76E-02 ± 0.18E-02** [1]	**6.03E-02 ± 0.10E-02** [1]
Thyroid	1.26 E−04 ± 0.2E-04	0.96 E−04 ± 0.3E-04	0.91E-04 ± 0.2E-04	**1.79E-04 ± 0.2E-04** [1]	0.87E-04 ± 0.1E-04	ND	1.01E-04 ± 0.1E-04	**01.93E-04 ± 0.4E-04** [1]
Thymus	2.30E-03 ± 0.09E-03	2.14E-03 ± 0.2E-03	2.08E-03 ± 0.2E-03	**1.85E-03 ± 0.1E-03** [1]	2.01E-03 ± 0.21E-03	2.06E-03 ± 0.11E-03	1.89E-03 ± 0.1E-03	1.78E-03 ± 0.3 E−03
Brain	1.10E-02 ± 0.09E-02	ND	**1.01E-02 ± 0.01E-02** [1]	1.06E-02 ± 0.03E-02	0.82E-02 ± 0.03E-02	ND	0.80E-02 ± 0.05E-02	0.80E-02 ± 0.03E-02

3.2.6. Histopathological analysis

Liver, kidney, thyroid, spleen, thymus, adrenals, and heart were all examined for histopathologic abnormalities in all animals in this study. Those organs which exhibited treatment-related pathology are discussed below. All other organs displayed no significant lesions attributable to treatment. A summary of the incidence and severity can be found in Table S3 in Supplemental Information.

3.2.6.1. Liver

Zone 3 (Z3) hypertrophy was significantly increased in incidence and severity in Fischer rats following treatment with A-HBCD (Table S3). Specifically, minimal Z3 hypertrophy in medium dose and mild Z3 hypertrophy in high dose females was observed. Mild Z3 hypertrophy was evident in three out of five animals in the Fischer male 1250 mg/kg diet group and all of the animals in the 5000 mg/kg diet group. An altered clear cell focus was noted in one male from the 1250 mg/kg diet group (Fig. 4).

3.2.6.2. Thyroid

A summary of thyroid lesions can be found in Table S3. Thyroids from the female 250 mg/kg diet group were unavailable for assessment. Follicular hypertrophy and hyperplasia were evident with increasing incidence and severity in a dose-dependent manner (Fig. 5). This hypertrophy and hyperplasia were coupled with colloid depletion in the two highest dose groups in both sexes. Colloid vacuolation was evident in all doses; however, colloid depletion in the 5000 mg/kg diet dose group rendered assessment of colloid vacuolation impossible.

3.2.6.3. Spleen

The incidence and severity of splenic lesions increased with increasing dose in both sexes (Table S3). In the Fischer females from the 1250 mg/kg diet dose, there was evidence of mild PALS and follicular atrophy while there was variable mild to marked PALS atrophy, coupled with mild follicular and MZ atrophy in the highest dose group. Male Fischer rats exhibited mild to moderate PALS and follicular atrophy in the 1250 mg/kg diet dose group, and moderate to marked atrophy in the 5000 mg/kg diet dose group (Fig. 6).

3.2.7. A-HBCD alters ovarian follicle population distribution

Evaluation of ovaries from Fischer rats exposed to A-HBCD enriched diet revealed a significant increase in the proportion of growing follicles, specifically the secondary stage of development (33.3 ± 1.97% vs 27.0 ± 0.97%; $p = 0.030$), in the high dose group compared to control animals (Fig. 7).

3.2.8. A-HBCD alters immunotoxicity parameters

Splenocyte proliferation was differentially decreased in males and females (Table S5). Specifically, at the highest dose, females showed a greater than 1.8-fold decrease in splenocyte proliferation irrespective of challenge; however, when normalized to unstimulated control, the effect was not significant. Moreover, unstimulated splenocytes also displayed decreased proliferation at this dose, indicating a generalized effect of A-HBCD on splenocyte viability. Males, on the other hand, showed decreased splenocyte proliferation in both LPS- and ConA-stimulated treatments at the two highest doses. Additionally, both male and female Fischers displayed an increase in serum immunoglobulin IgA at the high dose. In contrast to T-HBCD, treatment with A-HBCD elicited changes in both male and female Fischer blood lymphocyte populations. At the high dose, females displayed a decrease in T_H cells, an increase in T_C cells as well as a decrease in the T_H/T_C ratio but no change in the T cell/B cell ratio. Conversely, males showed changes in T cell and B cell populations at the highest dose, with decreases in T_H cells, T_H/T_C ratio and an increase in NK and B cells, leading to an

Table 9
Changes in haematology parameters following exposure to A-HBCD.[1] $p \leq 0.05$ relative to control.

HBCD added to diet (mg A-HBCD/kg diet)	Females				Males			
	0	250	1250	5000	0	250	1250	5000
Red blood cells (10^{12}/L)	7.78 ± 0.18	**8.03 ± 0.06** [1]	7.78 ± 0.12	7.71 ± 0.11	8.35 ± 0.21	8.43 ± 0.13	8.40 ± 0.12	8.33 ± 0.36
Haemoglobin (g/L)	144.8 ± 3.27	**149.0 ± 0.71** [1]	147.6 ± 2.41	144.8 ± 1.92	148.4 ± 2.19	152.2 ± 2.28	**156.0 ± 1.87** [1]	**157.8 ± 5.93** [1]
Mean corpuscular volume (fL)	51.40 ± 0.55	51.20 ± 0.45	51.40 ± 0.55	51.60 ± 0.55	52.20 ± 0.45	52.80 ± 0.45	53.60 ± 0.55	**54.60 ± 0.55** [1]
Mean corpuscular haemoglobin (pg)	18.56 ± 0.18	18.58 ± 0.16	**18.94 ± 0.27** [1]	18.80 ± 0.10	17.76 ± 0.27	18.04 ± 0.15	**18.58 ± 0.19** [1]	**18.92 ± 0.28** [1]
Mean corpuscular haemoglobin concentration (g/L)	361.00 ± 2.12	361.40 ± 1.52	**367.80 ± 3.03** [1]	**365.60 ± 2.97** [1]	341.20 ± 3.56	342.20 ± 1.92	**347.60 ± 1.34** [1]	**347.00 ± 3.94** [1]
Neutrophils (10^9/L)	0.24 ± 0.02	0.24 ± 0.09	0.25 ± 0.06	**0.39 ± 0.07** [1]	0.47 ± 0.12	0.57 ± 0.08	0.53 ± 0.09	**0.67 ± 0.09** [1]
Neutrophils (Ratio)	5.72 ± 0.82	5.52 ± 1.37	5.38 ± 0.88	**8.80 ± 0.70** [1]	7.56 ± 2.33	8.44 ± 0.73	7.46 ± 1.06	9.22 ± 1.70
Lymphocytes (Ratio)	92.40 ± 0.92	92.44 ± 1.67	92.44 ± 0.88	**87.60 ± 0.51** [1]	89.30 ± 2.39	88.50 ± 0.43	88.66 ± 1.68	86.46 ± 2.22
Monocytes (10^9/L)	0.058 ± 0.013	0.072 ± 0.033	0.082 ± 0.013	**0.128 ± 0.013** [1]	0.140 ± 0.043	0.180 ± 0.043	0.220 ± 0.034	**0.246 ± 0.075** [1]
Monocytes (Ratio)	1.38 ± 0.28	1.54 ± 0.36	1.74 ± 0.17	**2.94 ± 0.21** [1]	2.22 ± 0.55	2.60 ± 0.42	3.06 ± 0.34	**3.32 ± 0.64** [1]

overall decrease in the T cell/B cell ratio. Finally, while no changes in thymus lymphocytes were evident in females, males displayed a treatment-related effect at all doses in thymus lymphocyte subpopulations (Table S5).

3.2.9. BMD modelling

Although all study endpoints were modelled, not all endpoints could adequately be fitted to the BMD models. For those that fit the available models, BMDs and corresponding BMDLs are summarized in Table 12. In female rats, the lowest BMDLs were increased bicarbonate (BMDL 2.72 mg/kg BW/d), decreased magnesium (BMDL 7.71 mg/kg BW/d), and increased incidence of hepatocellular zone 3 hypertrophy, thyroid follicular cell hyperplasia and decreased area in splenic follicles (7.89 mg/kg BW/d, 4.90 mg/kg BW/d and 3.61 mg/kg BW/d, respectively). In male rats, the lowest BMDLs were decreased thymus CD4-8+ cell percentage (BMDL 3.30 mg/kg BW/d), decreased creatine kinase (BMDL 5.33 mg/kg BW/d), and increased incidence of hepatocellular zone 3 hypertrophy and decreased area in spleen PALS (7.26 mg/kg BW/g and 9.01 mg/kg BW/d, respectively).

4. Discussion

In this study, we investigated three rat strains treated with commercial grade (technical) HBCD or HBCD enriched with the α isomer to examine strain- and sex-related differences in response to exposure. As expected of the highly bioaccumulative HBCD, at the end of study all animals in all treatment groups had detectable, dose-dependent levels of HBCD isomers in their tissues. A sex-related difference in the hepatic HBCD concentrations was observed at all doses, irrespective of isoform, pointing to a better metabolising capacity for males than females. This finding is in keeping with those of others (Chengelis, 2001; Germer et al., 2006; Miller et al., 2016a; van der Ven et al., 2006, 2009; Yu and Atallah, 1980). We also observed a dose-dependent decrease in the ratio of γ/α-HBCD in all strains and both sexes, likely indicating an increased isomerization of γ-HBCD with increasing dose (Janssen, 2005; van der Ven et al., 2009) as well as a shorter half-life and the decreased ability of γ-HBCD to bioaccumulate (Szabo et al., 2010, 2011).

As noted in the results, the major isomers of HBCD in T-HBCD and A-HBCD given in diets were γ (98%) and α (81%), respectively. It was observed that the relative levels of the major isomers in tissues tested were significantly different between diet groups (A-HBCD vs T-HBCD). Female Fischer rats consuming A-HBCD high dose diets had adipose levels of total HBCD approximately 7.5 times the level of total HBCD in animals consuming T-HBCD high dose diets. Similarly, the ratio of A-HBCD/T-HBCD was 4.1 in liver and 8.7 in serum of female rats treated with high dose diets. In the tissues of A-HBCD treated rats, the α isomer constituted greater than 98% of the total isomers measured, while in T-HBCD treated tissues, it constituted between 10 and 13% of total HBCD. The presence of detectable α isomer in medium and high dose T-HBCD treated tissues support the bio-isomerization of γ isomer to α isomer as seen by Szabo et al. (2010). The γ isomer of HBCD constituted the remainder of the total HBCD detected. Similar ratios were observed in male rats treated at the highest dose, with the exception of liver where the A-HBCD/T-HBCD ratio of total HBCD detected was 11.1. These data indicate that the α isomer is preferentially taken up by the organism compared to the γ isomer. Single dose toxicokinetic studies with HBCD in mice (Szabo et al., 2010, 2011) have also shown sequestering of HBCD into liver, blood and adipose tissue as in the current study. Szabo et al. (2010) study found the liver accumulated the highest concentrations of γ isomer HBCD in contrast to the current study with serum and adipose were the prime reservoirs of γ isomer HBCD. The increased length of this study and higher dosing regimen maybe responsible for the increase in serum and adipose versus liver γ isomer levels, also the γ isomer levels in all three tissues plateaus in the medium dose, a possible indication of tissue saturation. Animals dosed with A-HBCD observed no such plateau across the doses, only increasing levels of HBCD. The levels of T-HBCD in this study were considerably higher in adipose and serum compared to liver by a factor of approximately 2 and 3 respectively.

HBCD exposure resulted in alterations to several organs, the most pronounced being to the thyroid in both sexes and in all strains. Given that HBCD is a suspected endocrine disruptor (Germer et al., 2006; Yamada-Okabe et al., 2005), it is unsurprising that the most pronounced effect of treatment was related to the thyroid. Since small, but significant changes to the function of the thyroid can result in transgenerational effects (Germer et al., 2006; Glinoer, 2001; Zhou et al., 2002), it is considered the most significant target organ of HBCD based on these and the results of others (Ema et al., 2008; Tung et al., 2016; van der Ven et al., 2006). Endocrine disruption through interference of thyroid homeostasis, is also considered the primary toxic effect of other BFRs, including tetrabromobisphenol a (TBBPA (Birnbaum and Staskal, 2004);). The thyroid is a sensitive target for xenobiotics owing to the multiple mechanisms by which alterations in its function can be achieved: histopathological alterations, changes in the synthesis of thyroid hormones, their breakdown, distribution, and/or binding to nuclear receptors. In our study, daily consumption of T-HBCD resulted in a decrease in brain weight in female Fischer rats, which could potentially be linked to impaired learning and memory (Eriksson et al., 2006; He et al., 2011). Perturbations in maternal thyroid function during development can result in changes in offspring, particularly neurodevelopmental changes (Birnbaum and Staskal, 2004). Thyroid weights were significantly increased in male Fischer rats following exposure to either of the test diets and in female Fischer rats following

Table 10
Changes in clinical chemistry parameters following exposure to A-HBCD.[1] $p \leq 0.05$ relative to control.

HBCD added to diet (mg A-HBCD/kg diet)	Females				Males			
	0	250	1250	5000	0	250	1250	5000
A/G Ratio	2.62 ± 0.15	**2.44 ± 0.11** [1]	**2.32 ± 0.08** [1]	**1.92 ± 0.08** [1]	2.06 ± 0.06	1.94 ± 0.06	1.98 ± 0.13	**1.82 ± 0.08** [1]
Albumin (g/L)	43.60 ± 2.70	44.00 ± 1.87	47.60 ± 2.79	**51.40 ± 1.34** [1]	41.20 ± 0.84	41.60 ± 1.14	**43.40 ± 1.14** [1]	**48.20 ± 0.45** [1]
Amylase (U/L)	1836.6 ± 114.6	1939.2 ± 119.6	**2304.8 ± 233.8** [1]	**3220.8 ± 364.8** [1]	2874.0 ± 123.6	3082.0 ± 109.8	**3232.6 ± 132.5** [1]	**3669.4 ± 185.0** [1]
Aspartate Transaminase (U/L)	80.40 ± 4.67	74.20 ± 6.10	**58.20 ± 6.34** [1]	**64.60 ± 5.51** [1]	84.20 ± 6.14	77.00 ± 6.44	**63.20 ± 5.40** [1]	**57.00 ± 3.00** [1]
Bicarbonate (mmol/L)	26.4 ± 1.67	27.80 ± 0.84	28.40 ± 1.14	**29.40 ± 1.14** [1]	27.0 ± 0.71	27.60 ± 1.67	**30.00 ± 1.23** [1]	**30.00 ± 0.71** [1]
Bilirubin (Total) (umol/L)	1.98 ± 0.33	**0.92 ± 0.42** [1]	1.60 ± 0.47	1.40 ± 0.12	1.62 ± 0.72	1.44 ± 0.67	1.34 ± 0.45	0.80 ± 0.16
Calcium (mmol/L)	2.76 ± 0.16	2.77 ± 0.09	2.92 ± 0.06	**3.15 ± 0.12** [1]	2.85 ± 0.03	**2.93 ± 0.05** [1]	**2.99 ± 0.06** [1]	**3.17 ± 0.04** [1]
Chloride (mmol/L)	99.6 ± 5.5	97.6 ± 1.8	97.0 ± 3.8	99.8 ± 4.2	98.0 ± 0.7	97.6 ± 1.5	101.0 ± 2.5	**108.4 ± 1.5** [1]
Cholesterol (mmol/L)	2.66 ± 0.26	2.54 ± 0.11	2.86 ± 0.20	**3.62 ± 0.25** [1]	2.28 ± 0.15	2.36 ± 0.23	2.32 ± 0.13	**2.74 ± 0.26** [1]
Creatine Kinase (U/L)	649.0 ± 49.0	**554.8 ± 16.0** [1]	**314.8 ± 50.5** [1]	**171.8 ± 35.1** [1]	718.8 ± 89.1	**572.4 ± 105.2** [1]	**347.8 ± 77.3** [1]	**165.4 ± 34.7** [1]
Creatinine (umol/L)	27.80 ± 4.55	25.80 ± 3.03	27.80 ± 6.18	29.20 ± 2.17	26.80 ± 2.28	22.80 ± 2.49	**21.60 ± 1.52** [1]	24.60 ± 2.19
Globulin (g/L)	16.80 ± 1.64	18.00 ± 1.23	**20.80 ± 1.79** [1]	**26.40 ± 1.52** [1]	20.20 ± 0.45	21.60 ± 0.89	**22.20 ± 1.30** [1]	**26.80 ± 0.84** [1]
Glucose (mmol/L)	10.14 ± 0.85	9.70 ± 0.74	10.40 ± 0.66	**8.76 ± 0.29** [1]	8.58 ± 1.21	7.96 ± 0.57	8.98 ± 0.83	10.12 ± 0.37
Lipase (U/L)	10.00 ± 2.35	10.20 ± 2.17	12.80 ± 3.11	**23.40 ± 5.64** [1]	15.00 ± 6.00	12.40 ± 1.82	16.00 ± 1.87	**23.20 ± 2.59** [1]
Magnesium (mmol/L)	1.07 ± 0.07	1.03 ± 0.04	0.98 ± 0.04	**0.96 ± 0.05** [1]	0.83 ± 0.04	0.78 ± 0.04	0.80 ± 0.03	0.82 ± 0.06
Sodium/Potassium Ratio	33.40 ± 0.55	33.40 ± 2.19	35.00 ± 1.23	**35.20 ± 0.45** [1]	33.40 ± 2.61	34.40 ± 2.07	35.00 ± 1.58	34.80 ± 1.10
Phosphorous (mmol/L)	2.70 ± 0.28	2.76 ± 0.25	2.82 ± 0.13	2.84 ± 0.18	2.82 ± 0.13	2.82 ± 0.08	**3.08 ± 0.16** [1]	**3.30 ± 0.16** [1]
Potassium (mmol/L)	4.30 ± 0.19	4.20 ± 0.33	4.00 ± 0.16	**3.84 ± 0.11** [1]	4.32 ± 0.38	4.14 ± 0.24	4.08 ± 0.19	4.12 ± 0.11
Sodium (mmol/L)	142.8 ± 6.7	140.0 ± 2.7	139.2 ± 3.8	**134.2 ± 3.6** [1]	144.0 ± 1.9	143.2 ± 1.1	142.8 ± 2.4	143.0 ± 1.2
Total Protein (g/L)	60.40 ± 4.28	61.80 ± 2.59	**68.80 ± 4.27** [1]	**77.60 ± 3.13** [1]	61.40 ± 1.14	63.20 ± 1.79	65.60 ± 1.82	**74.80 ± 1.30** [1]
Urea (mmol/L)	7.80 ± 0.68	7.00 ± 0.44	7.24 ± 0.29	7.94 ± 0.80	8.02 ± 0.80	6.98 ± 0.44	**6.78 ± 0.42** [1]	8.30 ± 0.88
Total T4 (nmol/L)	58.96 ± 9.81	61.98 ± 8.97	62.96 ± 8.63	**30.08 ± 3.99** [1]	100.12 ± 5.79	100.74 ± 10.05	**79.72 ± 5.13** [1]	**34.44 ± 0.90** [1]

Table 11
α and γ HBCD congener tissue concentration (ug/g) for female and male Fischer rats following exposure to A-HBCD.[1] $p \leq 0.05$ relative to control;[2] $p \leq 0.05$ relative to 250 mg A-HBCD/kg diet;[3] $p \leq 0.05$ relative to 1250 mg A-HBCD/kg diet. ND = not detected, limit of detection for α and γ in each tissue: serum 0.01 μg/g, liver 0.01 μg/g, adipose 0.02 μg/g.

	HBCD added to diet (mg A-HBCD/kg diet)	Females				Males			
		0	250	1250	5000	0	250	1250	5000
Alpha	Adipose	0.03 ± 0.01	**1080.92 ± 196.68** [1]	**1831.38 ± 140.678** [1,2]	**4632.93 ± 295.82** [1,2,3]	0.58 ± 0.11	**568.64 ± 28.11** [1]	**1448.05 ± 114.40** [1,2]	**3616.0 ± 106.67** [1,2,3]
	Liver	ND	**429.28 ± 77.75** [1]	**726.41 ± 100.55** [1,2]	**1225.02 ± 194.75** [1,2,3]	0.31 ± 0.05	189.13 ± 27.25	**537.03 ± 98.0** [1,2]	**1760.89 ± 364.05** [1,2,3]
	Serum	ND	**1864.97 ± 256.93** [1]	**2336.78 ± 263.24** [1]	**6750.79 ± 787.49** [1,2,3]	ND	649.31 ± 85.96	**1584.13 ± 258.72** [1]	**5385.47 ± 1434.87** [1,2,3]
Gamma	Adipose	ND	1.01 ± 2.25	ND	ND	0.06 ± 0.01	3.66 ± 8.19	ND	15.78 ± 35.28
	Liver	ND	ND	ND	ND	ND	ND	ND	ND
	Serum	1.30 ± 2.32	**41.46 ± 9.77** [1]	7.15 ± 16.0	ND	ND	21.84 ± 23.74	31.41 ± 29.19	**142.33 ± 82.81** [1,2,3]

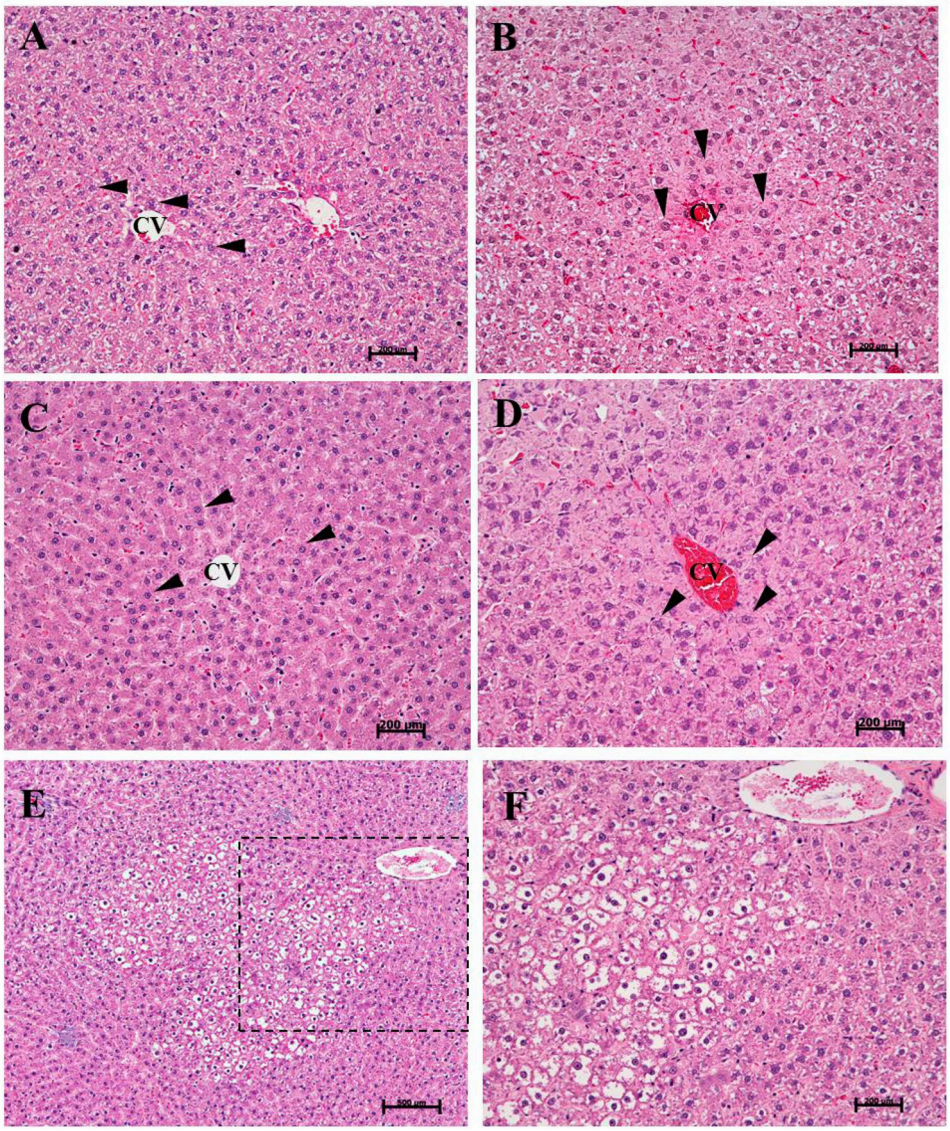

Fig. 4. Liver lesions in α-HBCD-treated Fischer rats. A and C) Control animals (female and male, respectively), B and D) 5000 mg/kg diet dose animals (female and male, respectively). Note hepatocyte hypertrophy in the 5000 mg/kg diet dose animals. Arrow heads: nuclei of hepatocytes; CV = central vein. E) Clear cell altered focus, 1250 mg/kg diet dose male, 10×, F) Clear cell altered focus, magnified from hatched section of in panel E, 20×.

administration of A-HBCD only, a probable indication of hypertrophy and hyperplasia, which could lead to the decreased T4 levels seen in our study. Sprague Dawley and Wistar rats showed no changes in thyroid weight following treatment. Contrary to our findings, a 28-day exposure to T-HBCD carried out in male and female Wistar rats reported that only the thyroids of females were increased in weight (van der Ven et al., 2006). While our study failed to show an increase in the thyroid weights of females exposed to T-HBCD irrespective of strain, increased thyroid weight approached significance for all three strains in the T-HBCD diet. Female Fischer rats in this study showed significant weight increases in the A-HBCD treated animals. This disparity could be due to differences in feed administration and strain (Turner et al., 2011). Furthermore the current study showed significant changes in histopathology of male Fischer thyroid treated with T-HBCD and female Fischer thyroid treated with A-HBCD, thus indicating a definite effect of HBCD on thyroid in multiple studies and multiple rodent strains.

Thyroid histopathology showed evidence of hypertrophy coupled with colloid depletion in a dose-dependent manner in both sexes and all strains tested in T- and A-HBCD-exposed rats, as well as decreased T4 and ALP levels and increased cholesterol in several rat strains and both sexes. Effects were more pronounced in A-HBCD-exposed females: dose-dependent increases in follicular hypertrophy and hyperplasia coupled with colloid depletion (C-A $p < 0.002$; Table S3) were accompanied by increases in thyroid weight and cholesterol and a concomitant decrease in total T4. Interestingly, increased phosphorus frequently occurred concurrently with decreased ALP, which is needed for cleaving oxygen and phosphorus from molecules for deposition in bones and teeth; lower ALP levels and changes in bone density have been associated with hypothyroidism (Tuchendler and Bolanowski, 2014). ALP is a marker of osteoblast activity and its decrease herein may be due to effects of HBCD on bone; which in turn is regulated by thyroid hormone signalling which is responsible for regulating bone development and ossification (Tung et al., 2016; van der Ven et al., 2006). Males exposed to T-HBCD had higher relative thyroid weights in the two highest doses, with follicular cell hypertrophy and colloid depletion that increased in severity as dose increased (C-A $p < 0.002$; Table S2). These adverse effects were more pronounced and included follicular cell hyperplasia and severe colloid depletion in A-HBCD-exposed males (C-A $p < 0.0001$; Table S3). In addition to the histopathological findings, T4 was decreased in male rats, with decreases occurring at a lower dose

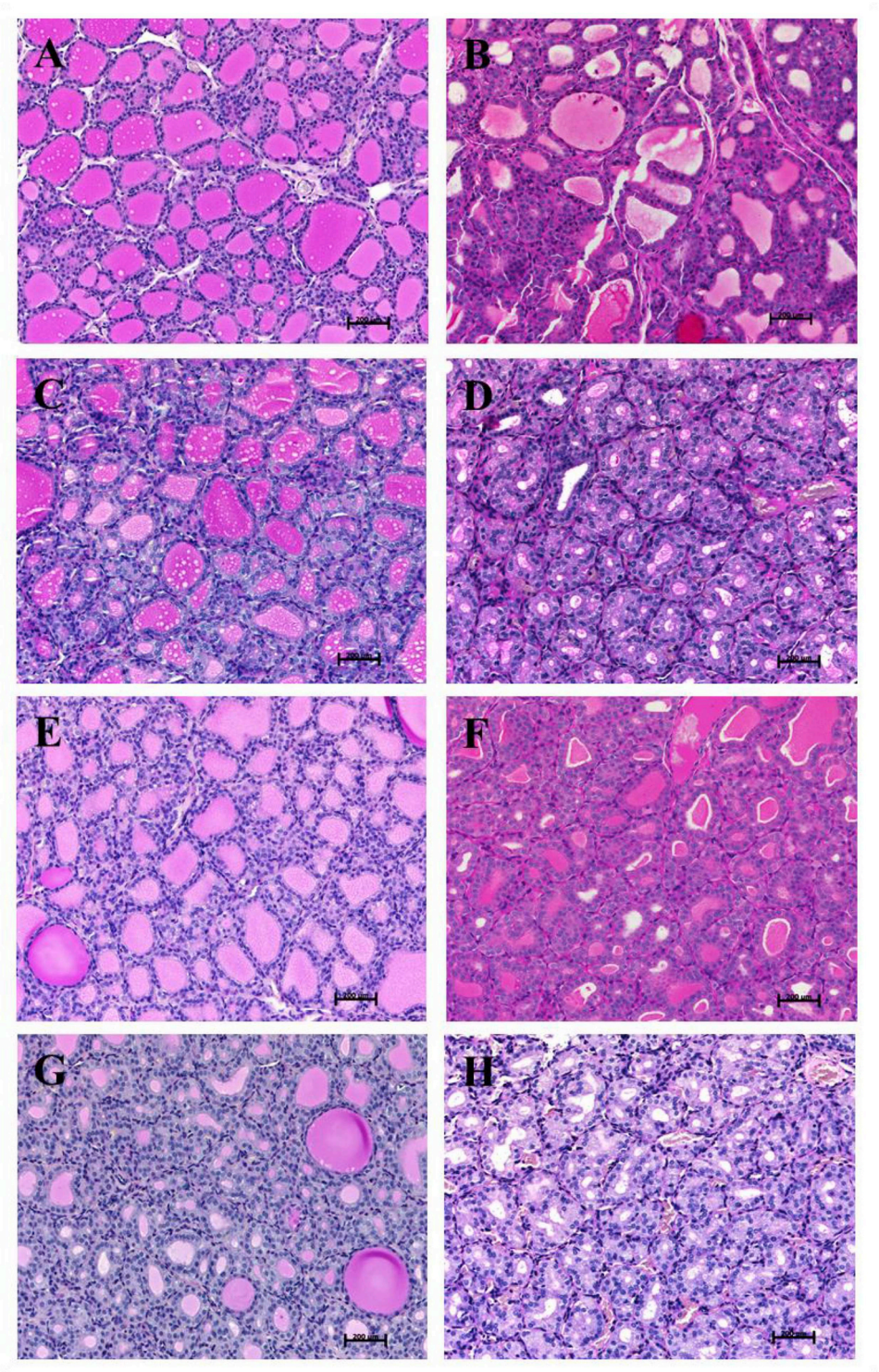

Fig. 5. Thyroid lesions in α-HBCD-treated Fischer rats. Female A) control, B) 250 mg/kg diet, C) 1250 mg/kg diet, D) 5000 mg/kg diet animals; Male E) control, F) 250 mg/kg diet, G) 1250 mg/kg diet, H) 5000 mg/kg diet animals. Marked dose-dependent colloid depletion and vacuolation is apparent in both male and female animals. Epithelial cell hypertrophy is noted in treated animals compared to controls. Magnification 20×.

in the A-HBCD-exposed rats. Serum cholesterol levels were elevated in the female rats for both diets, while male rat cholesterol levels were only elevated in the highest dose group of A-HBCD exposure. Proper thyroid function is required for the regulation of lipid metabolism, and diminished thyroid function has been linked to hypercholesterolemia (Canton et al., 2008) and is reflected in changed cholesterol levels.

Similar changes in thyroid weight, hypertrophy and colloid loss following exposure to T-HBCD have been reported previously (Chengelis, 1997; Zeller and Kirsch, 1969), although no other studies have looked at the effects of subacute exposure to A-HBCD. Increased thyroid weight and hypertrophy, coupled with decreased T4 levels, were also seen in both male and female rats treated with T-HBCD in a two-generation reproductive study (Ema et al., 2008), while Tung et al. (2016) also reported decreases in serum T4 and ALP levels and activation of pathways involved in thyroid regulation in pups exposed to T-HBCD *in utero* and during lactation. A recent study of an

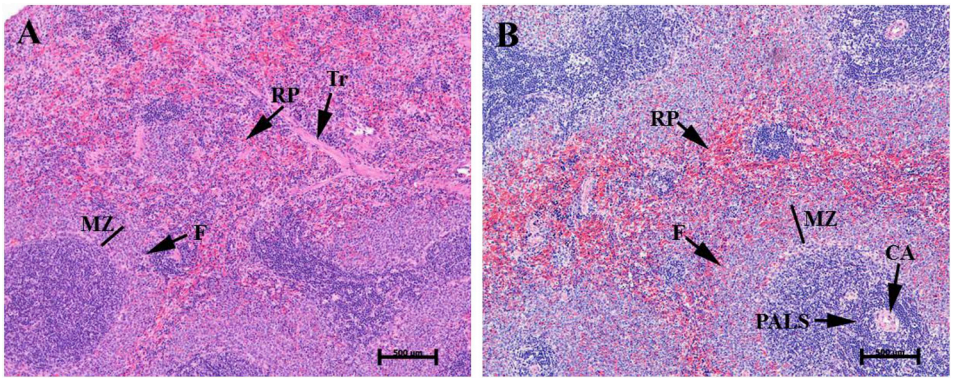

Fig. 6. Splenic lesions in α-HBCD treated male Fischer rats vs control. A) Control, B) 5000 mg/kg diet dose. Moderate to severe PALS atrophy is apparent in treated males. Marginal zone (MZ) is less dense than that of controls. CA = central artery; F = follicle; MZ = marginal zone; PALS = periarteriolar lymphoid sheaths; RP = red pulp; Tr = trabecula. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

environmentally-relevant BFR mixture, containing 3.3% HBCD, found altered thyroid function, including decreased serum T4 levels and changes in the morphology of epithelial cells of the follicles, suggesting that HBCD plays a role in the disruption of thyroid physiology (Ernest et al., 2012). In the present study, HBCD (irrespective of predominant isomer) evoked the greatest response in the endocrine system, most notably in the thyroid glands of both male and female Fischer rats, indicating the endocrine-disrupting potential of this BFR.

The pancreas was also affected by treatment with HBCD, with all three strains and both sexes showing altered parameters indicative of pancreatic damage (Hardt et al., 2009). The pancreas functions both as an endocrine gland, secreting insulin and glucagon to control blood sugars and as an exocrine gland, secreting various enzymes to aide in the breakdown of proteins, fats and lipids in food. The pancreatic enzymes amylase and lipase were significantly elevated following treatment at the two highest doses in both diets and sexes. Although all three strains of rats were affected by treatment, Fischers were the most affected, with alterations in levels of both enzymes, as well as increased triglycerides, at the two highest doses in T-HBCD-treated animals. Additionally, in female Fischers treated with A-HBCD, glucose levels were decreased in the highest dose group, pointing to a potential effect on the endocrine, as well as the exocrine, functions of the pancreas. Our findings are in contrast to other studies, which found no significant change in serum amylase, lipase or triglyceride levels following treatment with T-HBCD (Chengelis, 1997, 2001; Ema et al., 2008; van der Ven et al., 2006, 2009). They are, however, in agreement with van der Ven et al. (2006) and Chengelis (2001) with respect to changes in glucose levels.

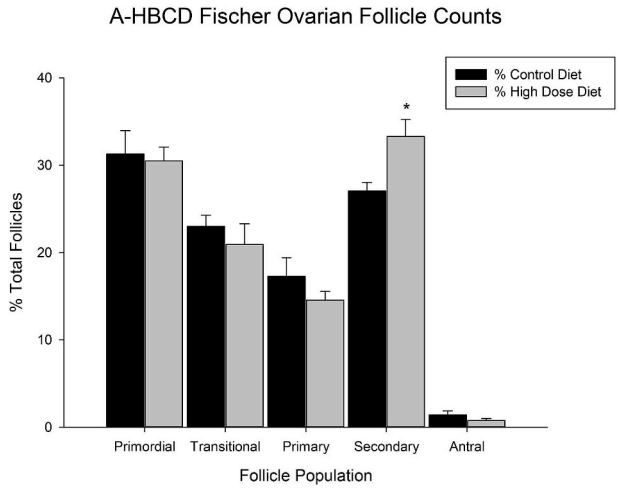

Fig. 7. α-HBCD treatment alters ovarian follicle distribution in Fischer rats. Ovaries from high dose and control Fischer rats were examined and the number of follicles at each stage of development was determined. Ovaries from rats fed a 5000 mg/kg A-HBCD diet had a significantly greater percentage of secondary follicles compared to control. *$p < 0.05$.

In view of the fact that HBCD is a suspected endocrine disruptor and that primordial follicles were depleted in the ovaries of the F1 generation of a two-generation T-HBCD study (Ema et al., 2008), ovaries were assessed in our study for changes in follicle population distribution following treatment with HBCD. Female Fischers were assessed as they were the most sensitive strain tested in other parameters in this study. Total number of follicles was not different between groups; however, population distribution (percent total follicles) was altered in the high dose animals in both technical- and α-exposed animals. Treatment with T-HBCD resulted in a decrease in the percent of growing follicles at the primary stage of development as well as a relative increase in the number of follicles in the antral stage of development (Fig. 3). Similarly, exposure to A-HBCD enriched diet resulted in disruption of ovarian follicle population distribution, notably an increase in the percentage of follicles at the secondary stage of development (Fig. 7). Although other reproductive endpoints for fertility were not measured in this study (cyclicity, fecundity, and litter sizes), Ema et al. (2008) found no differences in these parameters in either the F0 or F1 generations in a two-generation reproduction study, but given the relatively short exposure to HBCD, even in the two-generation study, it is possible that a chronic exposure could result in premature ovarian failure (POF). Exposure of experimental animals to certain environmental toxicants has been shown to destroy ovarian follicles, often in a stage-specific manner, resulting in POF (Devine et al., 2004; Jurisicova et al., 2007; Mayer et al., 2002; Tuttle et al., 2009). Thus, given sufficient time of exposure to HBCD, particularly α-enriched HBCD, as would be present in higher trophic level animals consumed as food, we would likely see similar effects on the reproductive lifespan of animals exposed to HBCD via diet. The decrease in the percentage of primary follicles following T-HBCD exposure and increase in that of secondary follicles following A-HBCD exposure suggests that the health of later stage follicles is affected, resulting in the recruitment of small follicles to compensate for adverse effects on the larger follicles. This is in keeping with other studies in rodent models of ovarian toxicants, namely 4-vinylcyclohexene diepoxide (Hoyer and Sipes, 2007), methoxychlor (Bhattacharya and Keating, 2012; Uzumcu and Zachow, 2007) and 7,12-dimethylbenz(a)anthracene (Madden et al., 2014), all of which elicit an increased recruitment of the small follicle population following a decline in the health of antral follicles, leading to POF (Lefevre et al., 2016). Our findings are also consistent with other BFR studies which found exposure to a mixture of BFRs, which included HBCD, designed to mimic levels found in house dust resulted in an increase in the number of preantral and antral follicles, and a correlation between the increase in those follicle populations and the number of atretic follicles therein (Lefevre et al., 2016). Furthermore, exposure to numerous flame retardants have been found to alter ovarian follicle oogenesis in previous studies, resulting in fewer mature and developing follicles (Ema et al., 2008; Zeller and Kirsch, 1969), alterations in estrogen, testosterone or progesterone secretion by ovarian follicles

Table 12
Benchmark doses and benchmark dose lower confidence limits for selected endpoints in Fischer rats exposed to A- HBCD in diet for 28 days.

Parameter[a]	BMD (range of BMDs for all acceptable models) in units of mg/kg bw/d	BMDL (range of BMDLs for all acceptable models) in units of mg/kg bw/d[b]
Fischer Females		
Increased relative liver weight (/BW)	43.40	36.56
Decreased relative thymus weight (/BW)	119.23 (109.76–210.70)	40.89 (21.93–156.40)
Decreased serum A/G ratio	67.50	47.59
Increased serum albumin (g/L)	103.24	75.09
Increased serum amylase (U/L)	28.87 (20.18–42.60)	18.59 (11.37–27.38)
Increased serum bicarbonate (mmol/L)	18.81 (18.81–195.75)	2.72 (2.72–128.33)
Increased serum calcium (mmol/L)	56.39 (56.39–103.28)	27.69 (27.69–76.55)
Increased serum cholesterol (mmol/L)	69.95 (69.95–88.06)	52.90 (52.90–62.12)
Increased serum globulin (g/L)	30.29 (30.29–74.39)	17.47 (17.47–57.86)
Increased serum IgA (10^3 ng/mL)	75.63 (37.29–75.63)	44.81 (27.52–44.81)
Increased serum lipase (U/L)	78.62 (54.40–78.62)	55.60 (36.01–55.60)
Decreased serum magnesium (mmol/L)	28.41	7.71
Decreased serum potassium (mmol/L)	232.34 (93.20–232.35)	152.63 (32.33–152.63)
Decreased serum sodium (mmol/L)	195.26 (134.75–198.24)	122.85 (13.67–126.32)
Increased serum total protein (g/L)	31.80 (31.80–78.36)	18.67 (18.67–58.73)
Decreased serum total T4 (nmol/L)	334.81 (178.05–334.81)	134.00 (118.99–157.09)
Decreased blood lymphocyte (ratio)	214.47 (82.96–214.47)	196.93 (63.94–196.93)
Increased blood monocyte (ratio)	57.26 (57.26–77.98)	43.84 (43.84–61.62)
Increased blood neutrophil (ratio)	234.35 (122.11–332.92)	128.88 (93.24–162.06)
Increased blood CD3+8 + cells (% lymphocytes)	167.33 (67.10–171.30)	111.44 (25.10–115.35)
Decreased blood CD4+:CD8+ ratio	110.33 (30.16–110.33)	79.52 (16.07–79.52)
Decreased unstimulated splenocyte blastogenesis ($x10^3$ dpm)	151.26 (119.05–163.76)	103.07 (49.92–103.14)
Decreased ConA-stimulated splenocyte blastogenesis ($x10^3$ dpm)	135.94 (117.27–235.77)	94.64 (61.98–99.09)
Decreased LPS-stimulated splenocyte blastogenesis ($x10^3$ dpm)	193.34 (142.18–229.93)	80.71 (76.59–91.36)
Increased incidence of hepatocellular hypertrophy, zone 3	67.70 (12.05–88.39)	7.89 (5.98–25.51)
Increased incidence of thyroid follicular cell hyperplasia	10.36 (10.36–87.20)	4.90 (4.90–19.27)
Increased incidence of decreased area in spleen follicles	8.19 (8.19–22.60)	3.61 (3.61–12.37)
Increased incidence of decreased cell density in spleen marginal zone	45.70 (45.70–130.41)	21.64 (13.72–66.16)
Fischer Males		
Increased relative liver weight (/BW)	92.85 (68.34–92.85)	84.94 (49.83–84.94)
Increased relative thyroid weight (/BW)	47.49	39.78
Increased serum albumin (g/L)	49.17 (34.78–53.48)	38.03 (21.97–41.55)
Increased serum amylase (U/L)	30.86	15.06
Increased serum bicarbonate (mmol/L)	18.81	8.86
Increased serum cholesterol (mmol/L)	164.34 (164.34–350.40)	112.18 (112.18–213.46)
Decreased serum CK (U/L)	9.00 (9.00–12.52)	5.33 (5.33–8.24)
Increased serum globulin (g/L)	59.15 (50.66–66.88)	45.42 (27.34–51.87)
Increased serum phosphorus (mmol/L)	36.40	19.45
Increased serum total protein (g/L)	44.91 (33.36–49.74)	34.83 (20.75–38.76)
Decreased serum total T4 (nmol/L)	30.56	21.98
Increased blood MCV (fL)	24.34 (19.35–106.31)	13.92 (9.00–78.37)
Increased blood monocyte ratio	222.22 (25.08–222.22)	140.69 (5.60–140.69)
Increased blood monocytes (10^9/L)	267.41 (26.01–236.38)	180.84 (4.73–180.84)
Decreased blood $CD3^+$ cells (% lymphocytes)	122.43 (51.89–128.29)	86.42 (24.03–91.65)
Decreased blood $CD3^+CD4^+$ cells (% lymphocytes)	134.83	102.29
Increased blood CD45RA + cells (% lymphocytes)	212.23 (108.85–223.29)	141.17 (43.49–153.09)
Increased blood CD45RA + cells (10^9/L)	161.81 (54.32–161.81)	118.17 (17.45–118.17)
Increased blood CD161a + cells (% lymphocytes)	52.02 (52.02–77.08)	23.07 (23.07–50.15)
Decreased blood T cell/B cell ratio	242.60 (106.12–242.60)	162.37 (43.19–162.37)
Decreased thymus CD4-8+ cells (% lymphocytes)	7.10	3.30
Decreased ConA-stimulated splenocyte blastogenesis (ratio unstimulated)	35.45 (35.45–94.42)	14.12 (14.12–45.21)
Increased incidence of hepatocellular hypertrophy, zone 3	66.53 (12.23–86.73)	7.26 (5.97–25.08)
Increased incidence of decreased area in spleen PALS	57.18 (8.33–83.33)	9.01 (3.93–18.72)
Increased incidence of decreased cell density in spleen follicles	25.23 (25.23–82.70)	12.94 (7.90–42.70)
Increased incidence of decreased area in spleen follicles	47.71 (47.71–141.36)	22.55 (12.90–72.06)
Increased incidence of decreased area in spleen marginal zone	32.29 (32.29–116.12)	15.90 (11.12–55.51)

[a] Abbreviations: A/G, albumin/globulin; BMD, benchmark dose; BMDL, benchmark dose 95% lower confidence limit; BMR, benchmark response; BW, body weight; CK, creatine kinase; ConA, concavalin A; MCV, mean corpuscular volume; PALS, periarteriolar lymphoid sheath; T4, thyroxine; WBC, white blood cells. Only results from data amenable to modelling by BMD software, as described in the Methods and Materials section, are included.

[b] All data generated in the study were modelled using US EPA's BMD software, version 2.5, as described in the Methods section. BMD and BMDL values are for the model of best fit according to criteria described in the Methods section; ranges are for all models with acceptable fit. Final BW, organ weight, and lesion incidence data were analysed using a default benchmark response (BMR) of 10% change in the mean, yielding a $BMDL_{10}$. For all other data the default BMR of one control standard deviation (SD) of the control mean was applied to the modelled endpoints, producing a $BMDL_{1SD}$. The lower limit of a one-sided 95% confidence interval localized on each parameter was used to derive the BMDLs in this table (EPA, 2012).

(Gregoraszczuk et al., 2008; Karpeta and Gregoraszczuk, 2010), aromatase activity changes (van der Ven et al., 2009) and altered mitochondrial morphology (Talsness et al., 2005). Taken together, our results suggest that HBCD is an endocrine disruptor in rats, affecting multiple endocrine organs, provoking complex actions on multiple endocrine-dependent pathways. This is particularly important in reproductive-aged females given that changes in maternal steroidogenesis can have lasting negative effects on the F1 generation's ovarian development leading to a decrease in primordial follicles (Ema et al., 2008), a process which is sensitive to steroid hormone levels.

The spleen and the peripheral immune system were also impacted by treatment with HBCD. The effects were more pronounced in male Fischer rats than in females of any strain, regardless of treatment diet. In rats exposed to T- and A-HBCD the spleens of affected animals exhibited decreases in the density and area of both the PALS and follicles and in the MZ. Splenocyte proliferation was significantly lower in stimulated (ConA and LPS) and unstimulated splenocytes from female Fischers exposed to T-HBCD and A-HBCD, and although the effect was not significant when stimulated groups were normalized to unstimulated, the decline in proliferation in unstimulated splenocytes suggests a decrease in viability due to treatment. Furthermore, Fischer males treated with T-HBCD and A-HBCD demonstrated decreased proliferation in unstimulated, ConA-stimulated (primarily T cell expansion) or LPS-stimulated (primarily B cell expansion) splenocyte cultures, suggesting a dampening in the adaptive immune response due to the inability of peripheral B and T cells to proliferate in response to a powerful mitogen.

Blood lymphocyte populations were also affected by both HBCD treatments, with decreases in T/B cell ratio resulting from a decrease in T cells (both T_H and T_C) and an increase in B cells and NK cells. Monocytes were elevated in all animals, except female Fischers, in the T-HBCD-exposed groups. Monocytes were also elevated in both male and female Fischers exposed to A-HBCD. This, coupled with increased total protein and increased NK cells, is indicative of an inflammatory response (Aggarwal and Pittenger, 2005; Heymann et al., 2015).

Thymus lymphocyte populations were unaffected in females; however, males were found to be sensitive to treatment with A-HBCD at all doses, indicating a generalized effect on the thymus. In the spleen, as with the thyroid, there appears to be a sex-related response to treatment wherein it seems to have an effect on female splenocyte viability, while in males A-HBCD alters the proliferation and population distribution of T and B cells, indicating an adaptive response to treatment. Our findings are in line with others who have found that treatment with T-HBCD resulted in perturbations of the immune system, including Tung et al. (2016), who found increased total protein in PND21 pups exposed to HBCD during gestation and lactation and van der Ven et al. (2006) who reported reduced T_H cell populations and dose-dependent increases in total protein in both male and female Wistar rats orally exposed to T-HBCD for 28 days. Similarly, in a two-generation reproductive study of T-HBCD exposure, total protein was significantly elevated in multiple treatment groups and carried forward into the F1 generation (Ema et al., 2008). A 90-day oral exposure study of HBCD in Sprague Dawley rats (Chengelis, 2001) also found that exposure resulted in increased total protein in both males and females, due primarily to increases in albumin in the males and globulin in the females – a phenomenon that was mirrored in our study. Our results are in contrast to others, however, who found no changes in haematological or clinical chemistry parameters (Chengelis, 1997; Ema et al., 2008; van der Ven et al., 2009), or no other changes with the exception of decreased splenic NK cell subpopulations (van der Ven et al., 2006). In all, histological changes in the spleen of rats exposed to T-HBCD and A-HBCD, and higher B cell numbers, particularly in male Fischers exposed to T-HBCD, may reflect deficits in immune function. However, in the absence of an effect on functional immunity (KLH-specific antibody production) in T-HBCD and A-HBCD-treated rats, the changes in splenic architecture and immune cell phenotype constitute equivocal evidence of immunotoxicity.

Given the role of the liver as a first-pass organ for the metabolism of xenobiotics, it is unsurprising that it is targeted by numerous BFRs, including HBCD (Birnbaum and Staskal, 2004; Chengelis, 1997; Darnerud, 2003; Ernest et al., 2012; Germer et al., 2006; Hakk, 2016). Moreover, previous studies have shown that higher levels of HBCD isomers (α and γ) are detected in the livers of females at necropsy following a 28-day (van der Ven et al., 2006) and 90-day exposure to T-HBCD (Chengelis, 2001). Our study found that treatment with either T- or A-HBCD resulted in elevated liver weights in female Fischers and Sprague Dawleys, while only treatment with A-HBCD resulted in appreciable changes in the liver weights of Wistars (for A-HBCD data, see Supplemental data Table S7). This is in contrast to a previous study in Wistars, which found a dose-dependent increase in liver weights in females exposed to T-HBCD (van der Ven et al., 2006), but in line with that of Chengelis (1997), whose study used Sprague Dawley rats. The discordant results may be due, in part, to differences in 1) administration of test chemical (diet vs gavage), and 2) doses employed (~400 mg/kg BW vs 200 mg/kg BW).

Analogous to van der Ven et al. (2006, 2009) and Chengelis (2001), the livers of female Fischers had higher residues of both HBCD isomers than those of their male counterparts, with a decreasing γ/α isomer ratio as dose increased. Although treatment with T-HBCD resulted in increased liver weights, no morphological changes were evident; however, A-HBCD-treated livers in both male and female Fischers exhibited hepatocellular hypertrophy in a dose-dependent manner (C-A $p < 0.001$; Table S3). This absence of morphological changes following T-HBCD exposure is in line with others (Chengelis, 1997, 2001; van der Ven et al., 2009), but contrary to those of van der Ven et al. (2006), who documented increased intensity of basophilia, an indicator of ER induction, in both sexes. Also different from van der Ven et al. (2006), our study did not find a significant decrease in ALP or an increase in total protein in female Wistars; nevertheless, there was a trend towards these changes, which could be due to difference in the doses administered. In Fischers though, we found that ALP is decreased in both males and females at all doses, and that total protein is increased in males at the highest dose for T-HBCD treated animals. These changes in total protein were seen in both males and females treated with A-HBCD. The decrease in ALP was curiously absent in A-HBCD treated rats, the cause of which is unknown and begs further investigation.

Interestingly, creatine kinase was consistently altered in all treatment groups for both diets and all three strains. CK, an enzyme whose increase is generally linked to muscle damage as seen in myocardial infarction, was paradoxically decreased in our study. Several studies have associated decreased CK with muscle wasting resulting from long-term immobilization due to critical illness (Gunst et al., 1998), yet the general health and activity of the animals in our study were not adversely affected by treatment. Contemporary studies of glutathione (GSH) metabolism indicate that CK expression levels are correlated with GSH levels. Endogenous GSH has been found to be a preserving agent of this enzyme while in circulation and therefore its depletion consequently results in a decrease in CK (Gunst et al., 1998; Jiang et al., 2007). Decreases in AST and ALP, indicators of B6 and B12 deficiencies, respectively, are both correlated with decreased GSH (Misra et al., 2017; Takeuchi et al., 1991). In male rats, decreased ALP, AST and CK are among the most sensitive parameters modelled for BMDLs. Their respective BMDLs (Tables 6 and 12) were: ALP (4.40 mg/kg BW/d), AST (4.68 mg/kg BW/d), and CK (14.45 mg/kg BW/d) for T-HBCD and 5.33 mg/kg BW/d for CK for A-HBCD. Similarly in females, AST and CK were among the more sensitive parameters modelled (AST: Fischer, 4.5 mg/kg BW/d; Sprague Dawley, 12.58 mg/kg BW/d; CK: Fischer, 4.65 mg/kg BW/d; Sprague Dawley, 15.14 mg/kg BW/d; Wistar, 5.30 mg/kg BW/d). Finally, others have found that treatment with HBCD resulted in decreased Gsta2 (Canton et al., 2008) and GSTP1

expression (Miller et al., 2016b). Glutathione-s-transferases, a class of proteins to which these belong, are themselves regulated by Nrf2 (Gorrini et al., 2013), which is in turn regulated by pregnane-X-receptor (PXR), which plays an essential role in xenobiotic metabolism and which has been shown to be induced following HBCD treatment of rat and human hepatocytes *in vitro* (Fery et al., 2010). Thus we postulate that HBCD is an agonist of PXR, thereby activating the signalling cascade that ultimately alters glutathione metabolism leading to decreased CK expression.

Effects on the liver have led to the determination of a LOAEL (lowest observed adverse effect level) of 13–925 mg/kg/day for T-HBCD (Birnbaum and Staskal, 2004; Zeller and Kirsch, 1970), while others have assigned NOAELs (no observed adverse effect level) ranging from 500 to 1000 mg/kg/day based on the lack of changes in a reproductive and developmental toxicity study (Murai et al., 1985) and the observed reversibility of the effects following 28-day and 90-day oral toxicity studies (Chengelis, 1997, 2001). Ema et al. (2008) estimated a NOAEL of 10 mg/kg BW/d based on a treatment-related decrease in fertility index in the F0 generation, a significant decrease in the number of primordial follicles in the ovary and a significant increased incidence of animals with decreased size of thyroid follicles in the two highest doses in both sexes in the F0 generation and the highest dose group of females in the F1 generation in a two-generation rat reproduction study. A screening assessment of HBCD by Environment Canada in 2012 considered this study to be the most relevant for characterizing the risk of HBCD to human health. A LOAEL of 0.9 mg/kg BW/day for assessing the risk of infants and children has been suggested based on significantly altered spontaneous behaviour, including hyperactive condition and reduced habituation, observed in mice following a one-time treatment with HBCD on postnatal day 10 (CMP, 2011; Eriksson et al., 2006).

In our study, histopathological and clinical evidence of changes in the liver, thyroid and spleen due to T-HBCD and A-HBCD were prevalent in the most sensitive rodent model, Fischer male and female rats. Since evidence of functional immunomodulation was equivocal, the liver and thyroid were considered to offer more robust choices on which to base a LOAEL/NOAEL or BMD/BMDL. For T-HBCD and A-HBCD, increased liver or thyroid weights in male or female rats were significant in the 1250 mg/kg dose group, producing LOAELs in the range of 100 mg/kg BW/d and NOAELs in the range of 20 mg/kg BW/d, taking estimated food consumption into account. Clinical changes such as decreased serum CK or ALP were significant in the 250 mg/kg dose group, resulting in estimated LOAELs in the range of 20 mg/kg BW/d and NOAELs of less than 20 mg/kg BW/d. Data modelling produced BMDs and BMDLs that were consistently lower than LOAELs and NOAELs. Notably, not all relevant data could be modelled so BMD/BMDLs were, of necessity, based on different parameters than LOAEL/NOAELs. Taking this into account, the lowest BMD/BMDLs for liver or thyroid changes in our study were 15.6 (BMD) and 7.6 (BMDL) for thyroid lesions in male rats exposed to T-HBCD, and 10.4 (BMD) and 4.9 (BMDL) for thyroid lesions in female rats exposed to A-HBCD. These are reasonably close to the NOAEL of 10 mg/kg BW/d of Ema et al. (2008) employed by the Canadian CEPA.

In conclusion, while the technical mixture employed here and in previous studies echoes that which is employed in the manufacturing of products, it does not reflect the chemical composition of HBCD isomers to which animals and humans are exposed via food. This is due to the propensity of the α isomer to bioaccumulate in the biota and food chain (Schecter et al., 2012; Xian et al., 2008) and the bio-isomerization of γ isomer to α isomer as seen by Szabo et al., (2010). It is important to note Szabo et al., (2017) indicated the isomer mixtures of HBCD given in a single dose may result in mixture-specific effects on HBCD metabolism resulting in different metabolites of HBCD, which may affect different biological pathways in the rat. While the three test mixtures had discreet metabolic profiles from one another, his study did not follow the generation of HBCD metabolites and therefore it is difficult to say what biological pathways are affected by specific metabolites. Our A-HBCD diet more closely reflects the real life distribution of isomers in tissue following long-term exposure, allowing for a better understanding of the potential risk from exposure to HBCD via food. The response to the technical diet mixture is suggestive of a short-term, initial exposure to HBCD; however, the A-HBCD diet is representative of a long-term, persistent exposure given the tendency for the α isomer to bioaccumulate in the biota. Residue analysis of tissues in our treated animals shows that while both α and γ isomers in the T-HBCD exposed animals approximated the same ratios as those in the diet at the highest dose in all tissues, the same is not true of the A-HBCD exposed animals. While the γ isomer comprised 12% of the A-HBCD-enriched diet, its levels were well below levels expected if ratios were to remain the same in the tissues of animals fed that diet, a fact that is likely due to the reduced bioaccumulative ability of the γ isomer as well as its propensity to isomerize into α in the body (Szabo et al., 2010). Total HBCD residues in tissues from A-HBCD diets accumulated at levels 4–12 times (dependent on sex and tissue) that seen in tissues from T-HBCD treated Fischer rats, with the majority of the increase due to the α isomer of HBCD. Moreover, we have shown here that differences in strains can have profound effects on the response to a chemical exposure, highlighting the importance of choosing the most appropriate and sensitive animal model when investigating potentially toxic chemicals. Our study also highlights the sex-related differences in response to exposure and reinforces the need for comparative studies between sexes in assessing the effects of chemicals.

Conflicts of interest

The authors report that they have no conflict of interest.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.003

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.003.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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