ASSESSING TOXICITY BENCHMARKS OF AROCLOR 1268 IN AMERICAN MINK (NEOVISON VISON), A SURROGATE MODEL FOR MARINE MAMMALS

By

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ABSTRACT

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Polychlorinated biphenyls (PCBs) from the commercial mixture Aroclor 1268 were released into the Turtle-Brunswick River Estuary (TBRE, southeastern Georgia, USA). Sum PCB concentrations in blubber samples from TBRE bottlenose dolphins (*Tursiops truncatus*) have been reported more than 10-fold those observed in adjacent regional estuaries and adverse effects have been suspected. Availability of toxicity data specific to Aroclor 1268 that are applicable to marine mammals are limited. Predicting toxic effects of Aroclor 1268 is uncertain due it's unique congener profile and associated physiochemical characteristics, such that data from other PCB mixtures may not be applicable. American mink were chosen as a surrogate model for cetaceans to develop mammalian PCB toxicity benchmarks. Mink are a suitable surrogate species for cetaceans in toxicity studies because of similarities in diet and taxonomic class. The mink's greater sensitivity to PCBs, compared to other mammals, allows for the application of mink toxicology data to provide a level of safety for cross-species extrapolations. A variety of ecologically-relevant effects in Aroclor 1268-exposed mink were assessed to support development of toxicity reference values for Aroclor 1268. Mink were exposed to Aroclor 1268 in diet at concentrations that ranged from 1.8 to 29 microgram (µg) Aroclor 1268/ gram (g) feed wet weight (ww), and the positive control contained 0.0009 to 0.0013 µg PCB 126/g feed ww.

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KEY TO ABBREVIATIONS

^{13}[C]	13-C isotope labeled
Ah	Aryl-hydrocarbon
AhR	Aryl hydrocarbon receptor
α	Alpha
°C	Degrees Celsius
CI	Confidence interval
CYP1A1	Cytochrome p450 1A1
EC20	Effective concentration resulting in a 20% response
EC50	Effective concentration resulting in a 50% response
EROD	Ethoxyresorufin-O-deethylase
g	Gram
GC-ECD	Gas chromatography-electron capture detector
GC-HRMS	Gas chromatography-high resolution mass spectrometry
HCT	Hematocrit
HGB	Hemoglobin
HDW	Hemoglobin distribution width
HxCDD	Hexa-chlorodibenzo-p-dioxin
HXCDF	Hexa-chlorodibenzofuran

HpCDD	Hepta-chlorodibenzo-p-dioxin
HpCDF	Hepta-chlorodibenzofuran
kg	Kilogram
LC20	Concentration resulting in 20% lethality
LC50	Concentration resulting in 50% lethality
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
mg	Milligram
MPV	Mean platelet volume
MSU	Michigan State University
ng	Nanogram
OCDD	Octa-chlorodibenzo-p-dioxin
OCDF	Octa-chlorodibenzofuran
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated dibenzofuran
PCB	Polychlorinated biphenyl
PeCDD	Penta-chlorodibenzo-p-dioxin
PeCDF	Penta-chlorodibenzofuran
pg	Picogram

PLT	Platelet count
pmol	Picomol
RBC	Red blood cell count
RDW	Red blood cell distribution width
SEP	Squamous epithelial proliferation
TBP	Total blood protein
TCDD	Tetra-chlorodibenzo-p-dioxin
TCDF	Tetra-chlorodibenzofuran
TEF	Toxic equivalency factor
TEQ	2,3,7,8-TCDD toxic equivalent
TRV	Toxic reference value
TBRE	Turtle Brunswick River Estuary
µg	Microgram
US	United States
WBC	White blood cell count
WHO	World Health Organization
ww	Wet weight

CHAPTER 1

INTRODUCTION

Polychlorinated biphenyls (PCBs) are synthetic compounds that were produced for industrial and commercial uses. These compounds were engineered for stability and inertness for use in heat transfer fluids, hydraulic fluids, flame retardants, plasticizers, waxes, and pigments among countless other applications (Erickson and Kaley, 2011). These molecules are also highly persistent in the environment when released as a result of their inertness and stability. Additionally, PCBs gravitate towards lipid rich phases such as adipose tissue and cellular membranes of organisms due PCB's low water solubility. These characteristics coupled with additional ecological and human health concerns led to the mandatory phasing out of PCBs for industrial and commercial uses in the United States (US) by 1979 (Giesy and Kannan 1998). Monsanto produced PCB mixtures under the trade name of Aroclor prior to 1979, and each Aroclor was designed with varying degrees of chlorination depending on their intended use. Aroclor 1268 was the most highly chlorinated of those mixtures, and has environmental relevance in the Southeastern US.

Sediments in the Turtle Brunswick River Estuary (TBRE, southeast Georgia US) are contaminated with Aroclor 1268, which is a PCB mixture dominated by octa-, nona-, and deca- PCB homologues (Kannan et al. 1997). Aroclor 1268 was not a widely used commercial PCB mixture, and its presence in the TBRE coupled with other possible PCB sources combine to make the type of exposure to wildlife within the TBRE unique (Kannan et al. 1998). Exposure to PCBs above threshold concentrations can induce adverse effects with regard to reproductive, immunological, and neurotoxic endpoints (Giesy and Kannan 1998). Animals occupying upper trophic levels are of particular interest because of the potential for PCBs to bioaccumulate through food webs. Pulster

and Maruya (2008) reported bottlenose dolphins (*Tursiops truncatus*) exhibit Aroclor 1268 contamination, and that homologues in dolphin adipose (blubber) tissue samples are consistent with 1268 exposure. On average, ∑PCB tissue concentrations are approaching those associated with the potential for adverse effects for lesser chlorinated and less hydrophobic PCB mixtures. However, the lack of toxicological data pertaining to this unique, highly chlorinated PCB mixture results in great uncertainty when trying to predict the toxicological potency, mode of action, and the site-specific potential for adverse effects (Kucklick et al., 2011, Pulster et al., 2009, Yordy et al., 2010).

Data pertaining to the toxicity of Aroclor 1268 are virtually negligible. Aroclor 1268 was not widely used, and relatively few instances of environmental release have been documented. The limited information that is available suggests that the relative potency of Aroclor 1268 could be more than an order of magnitude less than that of Aroclor 1254 (Kannan et al. 1998). This suggestion is consistent with the theory that PCB congeners associated with dioxin-like responses are the most likely to induce toxic effects (Giesy and Kannan 1998). Conversely, because Aroclor 1268 has the lowest relative concentration of dioxin-like congeners among commercial Aroclor mixtures it is plausible that toxic responses associated with alternate modes of action may occur prior to the predicted aryl hydrocarbon receptor (AhR)-associated response. Thus, a comprehensive, chronic dietary exposure study using an applicable surrogate species was necessary to isolate variables and test hypotheses. For this study we have selected American mink (*Neovison vison*) as the piscivorous mammalian model

because mink are known to be exquisitely sensitive to PCBs, they are well studied, they can be reared in the lab, and they are site-relevant.

The ubiquity of mink in aquatic environments, the mink's sensitivity to PCBs, and relative ease of husbandry for laboratory purposes all contribute to the species' appropriateness as a primary and surrogate species for toxicological modeling (Aulerich and Ringer 1977, Basu et al., 2007). Mink are historically the preferred surrogate for cetaceans such as bottlenose dolphins because the two have similarities in piscivorous diet, similar life history characteristics, and are members of the same taxonomic class (Kannan et al., 2000). The toxic reference values (TRVs) derived from this proposed study will be applicable to relevant species including the bottlenose dolphin, river otter, and wild American mink, with appropriate consideration of potential interspecies differences in sensitivity. Additional assets that can be employed include a large database of mink PCB exposure studies that have been conducted at the Michigan State University (MSU) Experimental Fur Farm (Shump et al. 1976, Aulerich et al. 1999).

This study was designed to provide mammalian toxicological data for Aroclor 1268. The study used mink as a piscivorous mammalian model and examined a wide array of measurement endpoints including morphologic, histologic, and reproductive parameters associated with chronic dietary exposures (Ringer et al. 1991). The goal of the study was to produce dose-response curves for mink reproductive endpoints that bracket exposures for bottlenose dolphins reported by Pulster and Maruya (2008). The resultant data are a significant improvement of the state of science pertaining to mammalian Aroclor 1268 toxicology. The purpose of the study described herein is to

discern if environmentally relevant concentrations of Aroclor 1268 are great enough to elicit adverse effects in a sentinel mammalian model (American mink). The mammalian model was chronically exposed to Aroclor 1268 through its daily diet over an entire reproductive cycle beginning prior to breeding and concluded at the sexual maturity of the offspring. Ecologically relevant measurement endpoints including survival and reproductive success and measures of individual health were quantified. This work provides novel data pertaining to Aroclor 1268 toxicity that can be used to assess risk to exposed wildlife.

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CHAPTER 2

GROWTH AND REPRODUCTIVE EFFECTS FROM DIETARY EXPOSURE TO AROCLOR 1268 IN MINK (*NEOVISON VISON*), A SURROGATE MODEL FOR MARINE MAMMALS

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ABSTRACT

Polychlorinated biphenyls (PCBs) from the commercial mixture Aroclor 1268 were historically released into the Turtle-Brunswick River Estuary (TBRE, southeastern Georgia, USA) from industrial operations. Sum PCBs (ΣPCBs) in blubber samples from TBRE bottlenose dolphins (*Tursiops truncatus*) have been reported at concentrations more than 10-fold higher than those observed in dolphins from adjacent regional estuaries. Given that toxicity data specific to Aroclor 1268 that are applicable to marine mammals are limited, predicting the toxic effects of Aroclor 1268 is uncertain due to its unique congener profile and associated physiochemical characteristics when compared to other PCB mixtures. American mink (*Neovison vison*) were chosen as a surrogate model for cetaceans to develop marine mammalian PCB toxicity benchmarks. Mink are a suitable surrogate species for cetaceans in toxicity studies because of similarities in diet and taxonomic class, and a characteristic sensitivity to PCBs provides a potential safety factor when using mink toxicology data for cross-species extrapolations. Effects of dietary exposure to Aroclor 1268 on reproduction, growth, and mortality in mink were compared to both a negative control and a positive control (3,3',4,4',5-pentachlorobiphenyl: PCB 126). Aroclor 1268 dietary exposure concentrations ranged from 1.8 to 29 micrograms Aroclor 1268 per gram feed wet weight (µg/g feed ww). Whelp success was unaffected by Aroclor 1268 exposure. Treatment mean litter size, kit growth, and kit survival were adversely affected relative to the negative control at various dietary ΣPCB concentrations of 10.6 µg/g feed ww and greater.

INTRODUCTION

Polychlorinated biphenyls (PCBs) were produced by Monsanto until 1979 under the trade name of Aroclor and designed with unique percent chlorination that depended on the intended use. Aroclor 1268 is the most highly chlorinated Aroclor, primarily composed of hepta- through deca-chlorinated PCB congeners (>98% by mass) and was used as a fire retardant and plasticizer among other applications (Kannan et al. 1997, Erickson and Kaley 2011). Polychlorinated biphenyls are environmentally persistent contaminants, and the potential for exposure is elevated in upper trophic level aquatic mammals due to the lipophilic and bioaccumulative nature of PCBs. Exposure to PCBs during reproduction may impair embryonic implantation and fetal development in mammals, resulting in spontaneous abortion, stillbirth, and low birth weight (DeLong et al. 1973, Reijnders 1986, Tanabe et al. 1994). Maternal transfer of PCBs to offspring, both *in utero* and via lactation is a major exposure pathway, affecting development and survival of offspring (Bleavins 1980, Aulerich and Ringer 1977, Reijnders 2003).

Aroclor 1268 was released into the Turtle-Brunswick River Estuary (TBRE) from a former chlor-alkali facility in Brunswick, Georgia, USA, which operated until 1994 (Kannan et al. 1997). Residues in blubber samples recently collected from bottlenose dolphins (*Tursiops truncatus*) in the TBRE exhibit PCB homologue composition consistent with Aroclor 1268 exposure (Pulster and Maruya 2008; Pulster et al. 2009). Mean sum PCB (ΣPCB) concentration in blubber of mature female TBRE dolphins was reported as 117 micrograms per gram lipid (µg/g lipid)(95% confidence interval [CI]=78.1-174, Balmer et al. 2011). Sum PCB concentrations in TBRE bottlenose dolphins exceed adverse effect thresholds derived from other environmental PCB mixtures and Aroclors. Kannan et al. (2000) proposed an adverse effects threshold for

marine mammals of 17 µg ΣPCB/g lipid in blubber based on field and laboratory studies of marine mammals and reasonable surrogate animal models. Reproductive impairment is of particular concern in primiparous dolphins. Based on a probabilistic risk assessment of bottlenose dolphins near the southeastern United States (US), Schwacke et al. (2002) proposed a 10% reproductive adverse effect concentration (EC10) of 14.8 µg ΣPCB/g lipid in blubber or liver. Sum PCB concentrations in some TBRE dolphins exceed both the Kannan et al. (2000) and the Schwacke et al. (2002) threshold effects concentrations, suggesting the potential for adverse reproductive effects. However, there is great uncertainty in the site-specific application of either of these threshold effect concentrations, given that they are based on PCB mixtures with greater relative abundances of the most toxicologically potent non-ortho-substituted (dioxin-like) PCBs relative to those present in the TBRE (Burkhard and Lukasewycz 2008, Heaton et al. 1995, Restum et al. 1998). The unique PCB composition and physiochemical properties of Aroclor 1268 can be expected to influence its relative toxicity and exposure potential (Kucklick et al. 2011, Pulster et al. 2009, Yordy et al. 2010). Threshold effects concentrations derived directly from Aroclor 1268 exposure studies and ecologically relevant endpoints thus would have greater relevance and less uncertainty; however, such data were lacking prior to this study. Available mammalian toxicity data for Aroclor 1268 were limited to acute neurotoxic and carcinogenic effects in rodent models (Simon et al. 2007, Warren et al. 2004) and thus were not applicable to the establishment of threshold effect concentrations for marine mammal survival and reproduction.

The greatest uncertainty associated with applying ΣPCB threshold effect

concentrations derived from other Aroclor mixtures to marine mammals exposed to Aroclor 1268 is the predicted difference in mixture potency related to dioxin-like toxicity. Dioxin-like PCBs are characterized as more potent than ortho-substituted (non-dioxin-like) PCBs in terms of aryl-hydrocarbon (Ah) receptor mediated toxicity (Giesy and Kannan 1998). Dioxin-like PCBs are less prevalent in Aroclor 1268 than in most Aroclor mixtures, suggesting that adverse effect thresholds for Aroclor 1268 could be considerably greater (i.e., less toxic) than those based on Aroclors 1254, 1248, and 1242. However, it is important to note adverse effect thresholds assume that most toxicity associated with these mixtures is mediated through the Ah receptor and that other modes of action are of lesser importance (Rushneck et al. 2004). Given these uncertainties, a study was conducted to assess the chronic effects of Aroclor 1268 on a broad scope of endpoints to account for multiple potential toxicity pathways.

Mink are the most appropriate surrogate toxicological model available for marine mammals within logistical boundaries. In contrast to rodents, both mink and marine mammals occupy upper aquatic trophic levels. Genomically, mink are more closely related to marine mammals than are rodents (Li et al. 1990, Higdon 2007). Genomic similarity inherited through common phylogeny suggests a likeness of adaptive physiological responses to contaminant exposure. This rationale is consistent with the strong homology of amino acid sequences of cytochrome-p450 enzymes CYP1A1 and 1A2 between mink and some pinnipeds as compared to rodents (Zhang et al. 2009). The function of CYP1A1/2 enzymes in PCB metabolism and a link between CYP450 enzyme activity and other Ah receptor mediated processes has supported the use of mink as surrogate models for marine mammals in previous risk assessments, and the

sensitivity of mink to PCB exposure arguably provides an inherent safety factor when extrapolating toxicity benchmarks from mink to marine mammals (Kannan and Giesy 2000, Schwacke et al. 2002, Basu et al. 2007). Here, adult female mink were continuously exposed to Aroclor 1268 through diet beginning prior to breeding, throughout the reproductive cycle, and continuing in the F1 generation. The effects of Aroclor 1268 on reproduction and development were evaluated in mink exposed to PCB concentrations relevant to those of TBRE bottlenose dolphins.

MATERIALS AND METHODS

Study mink and housing

Seventy second- and third-year adult, natural dark, female mink were randomly divided into 7 treatment groups of 10 individuals each and placed in breeder cages on December 29, 2011 to allow acclimation. Adult female mink were of known lineage and had produced successful litters in previous year(s). Siblings were excluded from treatment groups to eliminate genetic confounding variables. The Michigan State University Institutional Animal Care and Use Committee approved the use of all animals in this study and associated husbandry practices to ensure humane animal welfare (AUF# 11/11-230-00). Mink were maintained individually in steel wire cages (76 cm L × 61 cm W × 46 cm H) with front attaching nest boxes (38 cm L × 28 cm W × 27 cm H) lined with aspen shavings for nesting. The cages were positioned in adjacent aisles along the walls of an open-sided structure at the Michigan State University Experimental Fur Farm. Treatment groups were randomly assigned to blocks of 10 cages with an

empty cage separating treatment blocks. This arrangement prevented mink from being inadvertently exposed to unintended treatment diets from adjacent cages. Feed was administered each morning, and water was supplied ad libitum. Husbandry practices consistent with the "Standard Guidelines for the Operation of Mink Farms in the United States" were followed throughout the study (Fur Commission USA 2010).

Study design and feeding

The treatment diet PCB concentrations were designed to reflect concentrations of bottlenose dolphin preferred prey fish species collected from the TBRE and to achieve adipose PCB concentrations similar to those observed in TBRE female dolphins (Pulster et al. 2005, Balmer et al. 2011, Pulster et al. 2009). Seven treatment diets were administered to mink: a negative control (hereafter, "control"), 5 Aroclor 1268 treatments with increasing concentration, and a positive control diet spiked with PCB 126. Treatment diets were prepared in two rounds, the first in January 2012 and the second in June 2012. The initial batch of treatment diets was fed to adult female mink and kits until 6 weeks of age. Sum PCB concentrations were 1.77, 4.01, 10.6, 17.1, and 28.8 µg/g feed wet weight (ww). A second batch of treatment diets was mixed prior to weaning and was fed to juvenile mink from 6 to 27 weeks of age. Sum PCB concentrations of the second feed batch were 1.05, 3.10, 8.10, 16.8, and 28.8 µg/g feed ww. The first and second batches of positive control diets were spiked with 0.899 nanograms (ng) PCB 126/g feed ww and 1.31 ng PCB 126/g feed ww, respectively (Tables 1 and 2). Polychlorinated biphenyl 126 was selected as the positive control to evaluate mechanism(s) of action for Aroclor 1268 (see Folland et al. 2015).

Table 1. ΣPCB and ΣTEQ concentrations in diet, adipose, liver, and plasma by treatment for whelped adult female and 27-week-old juvenile mink

	Diet	Σ[PCB]		Σ[TEQ][a]	Adipose	Σ[PCB] Mean ± SD		Σ[TEQ][a]	Liver	Σ[PCB] Mean ± SD		Σ[TEQ][a]	Plasma	Σ[PCB] Mean ± SD	
	n	µg/g ww	µg/g lipid wt	pg/g ww	n	µg/g ww	µg/g lipid wt	pg/g ww	n	µg/g ww	µg/g lipid wt	pg/g ww	n	µg/g ww	µg/g lipid wt
Whelped females															
Control	1	0.0586	0.602	0.529	4	1.29 ± 0.287	1.89 ± 0.447	24.2	4	0.200 ± 0.0420	5.47 ± 1.54	1.44	10	0.145 ± 0.0901	20.8 ± 26.2
Aroclor 1268 1	1	1.77	20.3	7.69	4	112 ± 51.8	157 ± 73.5	207	4	9.41 ± 2.14	199 ± 39.2	56.8	7	1.7140 ± 0.701	212 ± 75.15
Aroclor 1268 2	1	4.01	40.3	14.9[b]	4	492 ± 305	492 ± 535	NA	4	26.0 ± 12.2	532 ± 185	NA	4	6.90 ± 3.23	708 ± 288
Aroclor 1268 3	1	10.6	102	35.1	4	649 ± 385	1330 ± 981	197	4	122 ± 10.6	4100 ± 656	106	4	12.7 ± 3.74	1470 ± 473
Aroclor 1268 4	1	17.1	175	52.2[b]	4	1610 ± 93.8	2350 ± 284	NA	4	171 ± 30.6	4690 ± 521	NA	4	16.4 ± 5.86	1610 ± 417
Aroclor 1268 5	1	28.8	282	85.1	3	1680 ± 344	2110 ± 657	615	3	298 ± 111	6330 ± 2120	176	3	22.9 ± 25.6	2580 ± 3120
Positive control	1	0.0717	0.700	90.2	4	6.14 ± 3.25	12.0 ± 9.83	1657	4	0.817 ± 0.600	16.0 ± 14.0	748	4	0.143 ± 0.0474	62.8 ± 97.4
% lipid	7	9.9 ± 0.58			27	64 ± 14			27	4.4 ± 1.1			36	0.89 ± 0.23	
27-week juveniles															
Control	1	0.059	0.602	0.529	4	0.0914 ± 0.00963	0.102 ± 0.0111	6.76	4	0.0602 ± 0.000682	0.118 ± 0.0183	8.98	3	0.0579 ± 0.000185	5.50 ± 1.64
Aroclor 1268 1	1	1.05	10.7	6.23	4	36.6 ± 5.44	40.1 ± 6.81	56.2	4	5.69 ± 1.05	116 ± 24.8	20.6	3	1.41 ± 0.369	114 ± 23
Aroclor 1268 2	1	3.10	28.8	12.3[b]	4	88.4 ± 14.2	98.6 ± 16.5	NA	4	12.9 ± 1.22	247 ± 36.1	NA	3	2.53 ± 0.230	249 ± 33
Aroclor 1268 3	1	8.10	77.0	26.6[b]	4	225 ± 101	266 ± 98.8	299	4	28.8 ± 9.25	579 ± 255	13.9	3	6.43 ± 2.09	527 ± 189
Aroclor 1268 4	1	16.8	164	51.4[b]	4	470 ± 131	526 ± 149	NA	4	55.0 ± 21.4	1080 ± 105	NA	3	13.0 ± 3.04	998 ± 146
Aroclor 1268 5	1	28.8	281	85.10	2	665 ± 17.1	735 ± 13.1	1306	2	56.1 ± 0.573	1260 ± 211	81.2	2	19.7 ± 1.20	1580 ± 186
Positive control	1	0.0687	0.629	131.5	4	0.0853 ± 0.167	0.498 ± 0.186	1278	4	0.0849 ± 0.00440	0.453 ± 0.124	1472	4	0.0582 ± 0.00100	5.65 ± 1.33
% lipid	7	10 ± 0.49			26	90 ± 6.2			22	5.8 ± 2.7			21	1.2 ± 0.20	

[a]Σ[TEQ] based on WHO_{2005} 2,3,7,8 TCDD TEFs from Van den Berg et al. 2006, n=1

[b]Dietary Σ[TEQ] value estimate based on linear relationship with Σ[PCB], not directly measured (see Supplemental Figure 1)

PCB: polychlorinated biphenyl

TEQ: WHO_{2005} 2,3,7,8 TCDD toxic equivalent

µg/g: microgram(s) per gram

pg/g: picogram(s) per gram

ww: wet weight

NA: Not analyzed

Table 2. ΣPCB concentrations in adipose, and liver by treatment for 6-week-old mink

	Adipose	Σ[PCB]		Liver	Σ[PCB]	
	n	**µg/g ww**	**µg/g lipid wt**	**n**	**µg/g ww**	**µg/g lipid wt**
6-week kits						
Control	4	0.187 ± 0.0577	0.312 ± 0.147	4	0.201 ± 0.168	0.297 ± 0.225
Aroclor 1268 1	4	25.1 ± 8.90	37.8 ± 18.1	4	4.16 ± 1.87	6.06 ± 2.52
Aroclor 1268 2	4	62.8 ± 19.4	81.5 ± 28.8	4	9.67 ± 1.04	12.5 ± 1.58
Aroclor 1268 3	4	144 ± 29.0	215 ± 42.2	4	122 ± 10.6	78.5 ± 18.7
Aroclor 1268 4	4	336 ± 310	925 ± 1310	4	83.4 ± 29.5	150 ± 28.5
Aroclor 1268 5	NA	NA ± NA	NA ± NA	NA	NA ± NA	NA ± NA
Positive control	4	0.119 ± 0.0233	0.856 ± 0.354	4	0.153 ± 0.0308	0.227 ± 0.0625
% lipid	24	66 ± 12		24	3.5 ± 1.1	

PCB: polychlorinated biphenyl
µg/g: microgram(s) per gram
ww: wet weight
NA: Not analyzed

Treatment diets were prepared based on an established formula that satisfies the nutritional requirements of mink (National Research Council 1982). Fish content was limited to a minimum amount of fishmeal to reduce the influence of potential co-contaminants. Chicken was substituted for fish to meet protein requirements. Each treatment diet was prepared separately in 272 kilograms (kg) quantities using a 455 kg capacity mechanical mixer. Feed was portioned into solvent-rinsed one gallon steel feed containers, then sealed, and stored at -25° Celsius (C). Diets consisted of 38 percent (%) water, 20% whole ground chicken, 16% GNF20 cereal mix (National Feeds), 6% spray-dried poultry liver (Van Elderen), 6% spray-dried egg (Van Elderen), 6% spray-dried Menhaden fish meal, 4% cheese (Michigan State University Dairy), 2% soybean oil (North American Nutrition), 2% spray dried blood protein (APC), and <1% vitamin and mineral mix (Akey), sodium bisulfate (Jones-Hamilton), biotin (Akey), and larvadex (Novartis). Treatment diets were spiked with Aroclor 1268 (Chemservice, Lot # 466-127A) or PCB 126 neat material (Accustandard, Lot # 082504MS-AC). The neat material was first dissolved in hexane and combined with soybean oil, after which the solutions were vortexed for several days until the volumes remained constant indicating the hexane had evaporated. The control diet was not spiked with PCBs.

Unexpected events required certain modifications to the study design and implementation. In January 2012, Aroclor 1268 dosing was initially attempted with treatments diets containing greater than 28.8 µg ΣPCB/g feed. Within the first day of treatment diet feeding, total food avoidance was observed at these higher PCB concentrations due to palatability issues. Several flavor additives used in mink feed in the past (beef flavor, beef liver, duck offal) were incorporated in small batches in

unsuccessful attempts to enhance palatability of treatment diets. By diluting treated feed with unspiked feed, it was determined that 28.8 µg ΣPCB/g feed ww was the highest concentration at which mink would consume an entire daily allotment. Mink that had consumed feed with PCB concentrations greater than this were excluded from the study.

Once the treatment diet concentrations were finalized, administration of the first round of treatment diets commenced in February 2012, one month prior to the beginning of breeding. Each mink was given 175 g of feed daily. Adult female mink were weighed at the commencement of the study and every three weeks after. Captive breeding took place in March 2012. Every eight days, a randomly selected male mink (unrelated to the female), was presented to each adult female mink until three successful copulation events were observed, to ensure an adequate opportunity for insemination. Pregnant females (dams) were not weighed during gestation to minimize stress during this sensitive time period. Dams gave birth to (whelped) kits between April 23 and May 9, 2012. Dams and kits were weighed, sexed, and examined for morphological abnormalities 24 hours after whelp, when kits reached 3 weeks of age, and at weaning when kits reached 6 weeks of age. Necropsies of dams and a subset of kits were performed at weaning. Ten kits per treatment were selected for necropsy, and an additional 10 kits were selected for continuation of treatment diet feeding. Kits were randomly selected to maximize representation of litters in each treatment group. One kit per litter was selected when available. Selection for continuation of treatment diets was given preference over necropsy for treatment groups with whelp success less than 100%. Necropsy and tissue collection of all dams and kits were performed June 19-27,

2012. Remaining kits were archived as whole body samples and stored at -25 °C. The second batch of treatment diets was administered to weaned kits (juveniles) until sexual maturity at 27 weeks of age when the exposure period concluded with necropsy. Masses of juvenile mink were recorded every four weeks. Juvenile necropsies were performed November 6-8, 2012.

Reproductive measurements

Gestation duration was defined as the number of days between the last confirmed copulation and whelping for each female. Whelp success was defined as the number of dams per treatment that whelped at least one live kit divided by the total number of dams. Total litter size describes the number of live and stillborn kits present at whelp, while live litter size excludes stillborn kits. Mortality endpoints were also assessed. Kit survival at whelp was calculated based on the number of live and stillborn kits in each litter. Treatment differences in lifespan of both total kits whelped and live born kits from whelp to 6 weeks and to 27 weeks were also measured.

Necropsy and tissue collection

Prior to necropsy, whole blood was collected from dams and juvenile mink for plasma PCB analysis and thyroid hormone quantification in serum (see Folland et al. 2015). Blood collection was not performed on kits due to insufficient blood volume for analysis. Mink were anesthetized via intramuscular injection with 1 milliliter (mL) /kg body weight of ketamine (Veterinary Products), abdomens were shaved, and 25 mL of blood was collected in Vacutainers® by cardiac puncture. Mink were euthanized by

carbon-dioxide (CO_2) asphyxiation immediately following blood collection. Vital signs were checked to confirm expiration. Separate solvent rinsed instruments were used for each mink to avoid cross-contamination. Liver, kidneys, spleen, heart, thymus, brain, uterus, jaws, and adrenal and thyroid glands were removed, massed, and examined by a board certified pathologist for gross abnormalities. Organs were stored in 10% neutral buffered formalin for histopathological examination described in a concurrent publication (Folland et al. 2015). All samples for analytical chemistry were collected and stored in certified chemically clean glass jars and stored at -25°C until contaminant analyses could be performed.

Analytical chemistry

Polychlorinated biphenyl, polychlorinated dibenzo-p-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) extraction (modified from EPA Methods 8082, 1668, and 1613): Diet, tissue, and plasma samples were analyzed with gas chromatography-electron capture detector (GC-ECD) for a suite of congeners intended to represent those found in Aroclor 1268 as well as 50 additional congeners of environmental significance. All treatment diets, liver and adipose from 4 mink per treatment and age class (where available), and plasma samples from 3 mink per treatment and age class were analyzed by GC-ECD. A subset of those dietary and tissue samples were analyzed for PCDDs/PCDFs and non-ortho PCBs so that exposures could be quantified in TEQs (Table 1).

Polychlorinated biphenyls for analysis by GC-ECD were spiked with [^{13}C] PCB surrogate standards (Accustandard) and extracted by soxhlet method in 400 mL 3:1

dichloromethane: hexane over 18 hours. Extracts were concentrated by evaporative nitrogen stream and dehydrated with anhydrous sodium sulfate. Extracts were streamed with nitrogen gas until dry, and lipid contents were weighed. Lipids were reconstituted in hexane and hydrolyzed with sulfuric acid in separatory funnels. The contents were then cleaned with acidic/neutral silica gel columns and concentrated to one mL in hexane. Samples for PCDD/PCDF and non-ortho PCB analysis by gas chromatography-high resolution mass spectrometry (GC-HRMS) were spiked with [^{13}C] PCDD/PCDF and dioxin-like PCB surrogate standards (Wellington). Polychlorinated dibenzo-p-dioxins, PCDFs and non-ortho PCBs were extracted in 400 mL of toluene using the same soxhlet extraction protocol as samples for GC-ECD analysis with the exception of a final carbon column cleanup step. Extracts were eluted through activated carbon/ silica gel and washed with hexane to remove ortho-substituted PCBs. The carbon bed was then slowly eluted with toluene. The PCDDs, PCDFs, and non-ortho PCBs collected in this elution were concentrated by rotary evaporation, quantitatively transferred to 250 µL microvials, and further concentrated in 20 µL of tetradecane. Laboratory blanks were included in each 12-sample extraction batch, and quality assurance/ quality control (QA/QC) samples including laboratory control sample, matrix spike, matrix spike duplicate, and standard reference material (SRM) samples were included every 20 samples. Lake Superior fish tissue and whale blubber (National Institute of Standards and Technologies) were used as SRMs.

PCBs by GC-ECD (EPA Method 8082): Sixty-one PCB congeners were quantified in diet, adipose, liver, and plasma sample extracts by GC-ECD (Hewlett Packard 5890 Series II) at the MSU Wildlife Toxicology Laboratory using a modified protocol of EPA

Method 8082. Sum PCB concentrations were calculated by adding the concentrations of 61 congeners (Table A1). When calculating ΣPCB concentrations, non-detect results and results below the method detection limit (MDL) were adjusted by applying a standard proxy value of one-half the congener specific MDL. Concentrations are expressed in ww unless noted otherwise.

PCDDs, PCDFs, and non-ortho PCBs by gas chromatography-high resolution mass spectrometry (GC-HRMS, modified from EPA Methods 1613 and 1668): Seventeen PCDD/PCDF and 4 non-ortho PCB congeners were quantified in sample extracts by high resolution gas chromatography-mass spectrometry (Thermo Scientific™ Ultra Trace) with a ZB-5MSi Zebron® capillary column (30 m×0.25 mm×0.25 µm) (Phenomenex; Torrance, CA, USA) at the Wadsworth Institute (Albany NY, USA). Non-ortho PCB analysis followed EPA Method 1668, and PCDD/PCDF analysis followed EPA Method 1613. Sum TEQs were calculated by summing the products of each PCDD/F and dioxin-like PCB congener and their associated 2005 World Health Organization mammalian Toxic Equivalency Factor (TEF) (Van den Berg et al. 2006; Table A1). Because MDL adjustment of non-detect results for PCDD, PCDF, and non-ortho PCB concentrations had a negligible effect on the calculated ΣTEQ, non-detect results were assigned a value of zero. A strong linear relationship existed between measured ΣTEQ vs ΣPCB concentrations in diet samples (R^2>0.999). This allowed ΣTEQ values to be estimated for dietary samples not directly measured by GC-HRMS (Figure A1). Such estimations were deemed inappropriate for tissue samples based on apparent dose-dependent differences in toxicokinetics, and only measured ΣTEQ values are reported for tissues.

Statistical analyses

All statistical analyses were performed using Statistical Analysis Software (SAS version 9.4, SAS Institute Inc.) with the exception of growth and mortality dose-response curves used for effect and lethal concentration (ECp/LCp) analyses, which were conducted using R (version 3.0.3). Endpoints were classified as continuous, count, or binary and assessed using two strategies. Overall effects from PCB exposure on continuous and count endpoints were evaluated using generalized linear model regression, and treatment differences from control were established by one-way fixed effect analysis of variance (ANOVA) using Dunnett's Test for least-squares means differences (PROC GLM). Where methods permitted, litters (as opposed to individuals) were treated as replicates to avoid pseudoreplication. Linear regression model assumptions of linearity, normality, homoscedasticity, and independence were tested systematically to determine appropriate application of the model. Linearity and normality of response variable distributions/residuals were examined graphically and tested using the Shapiro-Wilk statistic (Alpha [α]=0.05). Box-Cox power transformations (PROC TRANSREG) were applied when deviation from normality was detected (Lambda [λ] restricted from 3 to -3). Levene's Test for heteroscedasticity (alpha [α]=0.05) was used to assess heterogeneity of variances, and weighted least squares linear regression was applied where this assumption was violated. Independence was tested using the Durbin-Watson statistic. Application of the linear model and reporting of significant results were contingent on satisfaction of model assumptions. Nonparametric Kruskall-Wallis one-way ANOVA (PROC NPAR1WAY) was used to

identify treatment differences from control for continuous variable endpoints that could not be reasonably transformed to meet normality assumptions. Count variables were analyzed by log distribution-linked Poisson regression using PROC GENMOD with maximum likelihood odds ratios used to deduce treatment differences. Binary variable types were fit to binomial distributions, and treatment differences were assessed using Fisher's exact test (PROC FREQ). Error bars in figures represent one standard deviation (SD), unless other wise noted.

Body condition index (BCI) was used as a surrogate metric for nutritional state. The index was calculated by plotting body mass (g) vs body length (head to base of the tail, cm) for each age class. The value of the residual difference in body mass (y) from the body mass vs body length ordinary least squares line of best fit is the individual's BCI value (Schulte-Hostedde et al. 2001; 2005). Individuals with negative BCI values (less than the line of best fit) were assumed to be in a compromised nutritional state. Body condition index for dams was calculated using body masses measured the day after whelp. This time point provided the best representation of the nutritional state of dams at the beginning of lactation, when growth and survival of offspring are most dependent on maternal energetic reserves and differences in body mass losses from lactation among dams had not yet been greatly affected by litter size. Increasing body length during growth dictated that juvenile BCI calculations were based on masses recorded at necropsy. Body condition indices were not calculated for kits because the 6-weeks of age time point coincides with a rapid growth phase when body lengths of individuals may not be comparable, so the BCI value of each kit's respective dam was substituted as a surrogate metric.

The occurrence of infanticide in the study required a bias-minimizing approach to estimating kit mortality. The PROC LIFEREG procedure for survival analysis of right-censored data was employed to allow for the inclusion of kits subject to infanticide in survival estimates before the mortality event and the censoring of data points post-infanticide. This approach has been used for analyzing mortality in animal toxicity studies to eliminate bias in the presence of confounding factors (Anderson et al. 2000). The influence of BCI was assessed in endpoints that correlated significantly with PCB exposure or exhibited treatment differences. Mixed model statistics are typically the preferred approach for addressing multicollinearity, but were not appropriate here due to the non-independent relationship between covariates. Instead, correlations between response variables with BCI were tested, as well as correlations between the response variables and PCB concentrations excluding individuals with negative BCI values or treatments with mean BCI less than control. When response variables correlated with BCI or previously significant relationships were no longer true when BCI compromised individuals/treatments were excluded, nutritional status was deemed a cofactor. These relationships are reported for applicable endpoints.

Dose-response curves for kit mortality at 6 weeks, kit mass at 6 weeks, and juvenile length at 27 weeks were modeled based on diet, adipose, and liver ΣPCB concentrations. Maternal tissue concentrations were used for kit growth and mortality analyses, while juvenile effect concentrations were derived from PCBs measured in tissues of juvenile mink. Models were selected based on comparisons of the lowest Akaike Information Criterion values as well as visual and conceptual fit. A beta-binomial model was selected for kit mortality (Williams 1975, Crowder 1978, and Prentice 1986).

Linear models achieved the best fit for the selected growth endpoints. While linear models were the best predictors of kit mass and juvenile length, there was a recurring non-linear (hormesis-like) relationship at relatively low doses that resulted in biased variance estimates for the highest Aroclor 1268 treatments. Accuracy at the higher doses provides greater utility than the lesser treatments since most adverse effects on growth were observed in the greater treatments. To maximize model fit at the dietary and tissue concentrations of greatest interest, the Delta Method was used to estimate variances and calculate regressions. This is a nonparametric method designed to minimize influence of nonlinear parts of otherwise linear regressions. By uncoupling the variance of the control group from the regression calculation, bias from the nonlinear part of the regression at the low end of the curve and increases curve fit in the area of the regressions were most effects were observed (Efron 1981, Oehlert 1992, and Papke and Wooldridge 2005). Control adjusted 20 and 50% effect concentration (EC20/EC50) estimates were calculated growth endpoints. These benchmarks are described as lethal concentration estimates (LC20/LC50) for kit mortality.

RESULTS

Multiple factors resulted in reduced samples sizes for certain dose groups and age classes. Several weeks after the final dosing regime was established, decreased feed consumption was observed in the 17.1 and 28.8 µg ΣPCB/g feed ww groups, presumably due to the palatability issue described previously. Mink in these groups would consume control feed when it was presented and then would refuse feed when Aroclor 1268 exposure was resumed, suggesting that PCB-related palatability and not

wasting syndrome was driving decreased feed consumption (Aulerich and Ringer 1979, Aulerich et al. 1985, Hochstein et al. 1998). Six of the 10 adult females in the 28.8 µg ΣPCB/g feed group could not be sustained beyond 4 weeks in accordance with the study-specific animal care and use guidelines, and were subsequently euthanized. The sample size of this group was decreased to 4. Although all treatment groups were maintained, the nutritional state of individuals in the two highest groups was compromised. With the exception of the 4.01 µg ΣPCB/g feed ww group, the occurrence of infanticide in all Aroclor 1268 treatments and the positive control also had an adverse effect on sample sizes. Also, one pregnant female mink in the 1.77 µg ΣPCB/g feed ww group died during whelp from an obstructed birth canal. This individual was omitted from all analyses with the exception of adult female body masses prior to the mortality event, resulting in a decreased sample size of 9 in that group. Two juveniles (in the control and 8.10 µg ΣPCB/g feed groups) also died from heat stroke during an uncharacteristic 14 day period of >38°C temperatures in July of 2012.

Analytical chemistry

Sum PCBs and ΣTEQs in adipose, liver, and plasma of adult and juvenile mink are summarized in Table 1, while ΣPCBs in adipose and liver of kits are given in Table 2. Polychlorinated biphenyls 206, 203, and 201 contributed most to dietary PCB congener profiles, comprising 60-62% of ΣPCBs (Figure 1).

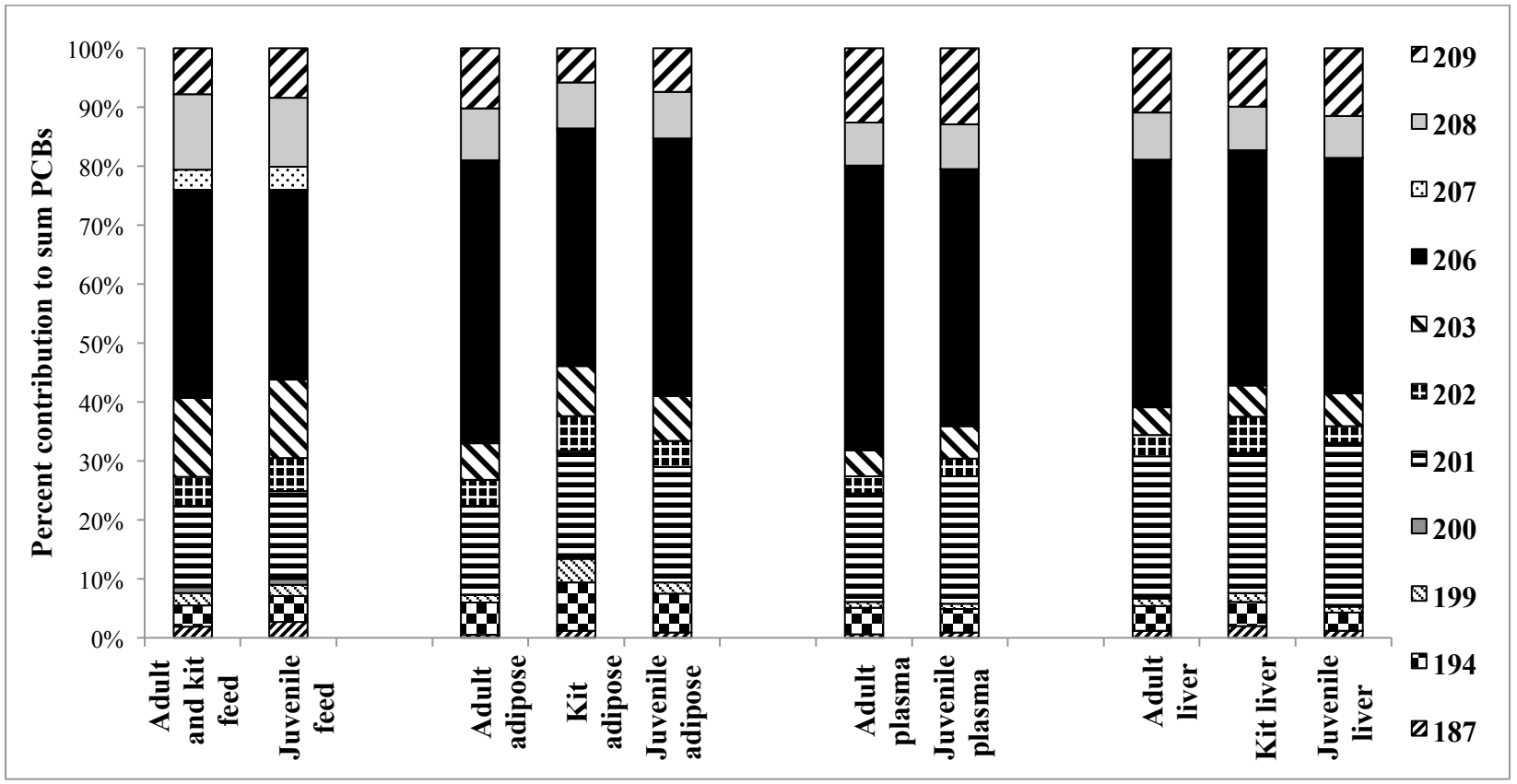
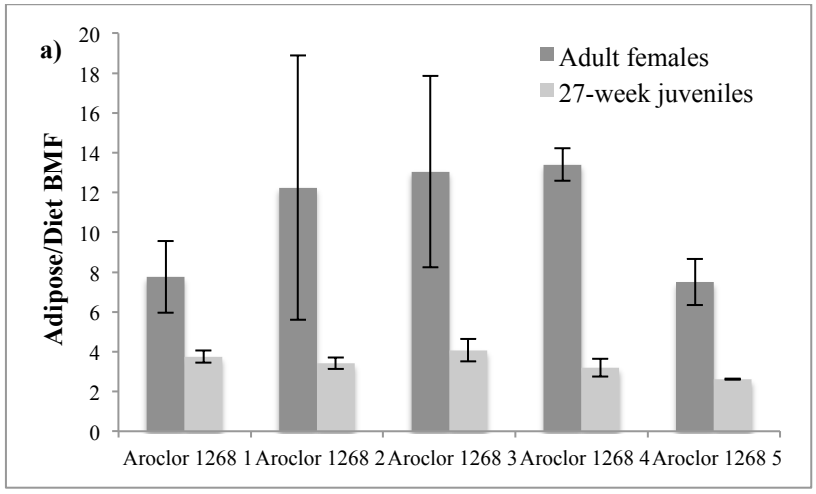


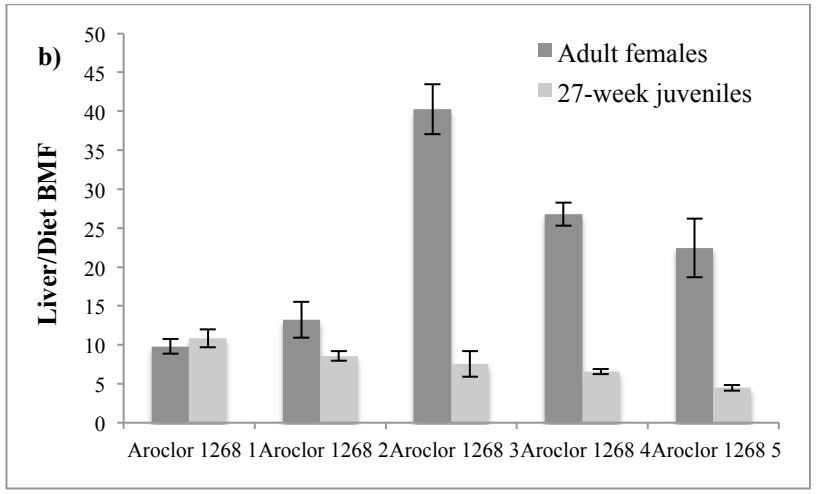
Figure 1. Relative abundances of PCB congeners in diet and tissues by age class

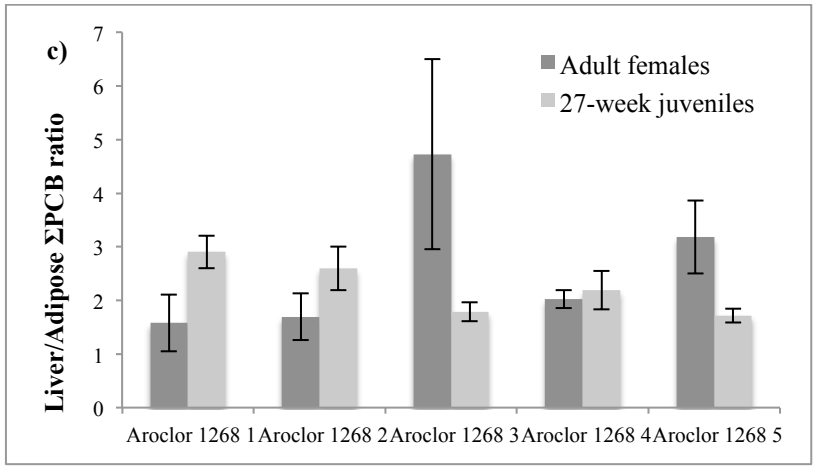
Sum PCB concentrations in the treatment diets administered to adult female and kit mink from the first batch of feed were slightly greater than those in the second batch of feed given to juvenile mink (Tables 1 and 2). Sum PCB concentration in adipose tissues, hepatic tissues, and plasma of Aroclor 1268-exposed adult female, kit, and juvenile mink were highly correlated with dietary PCB concentration ($p<0.001$, $R^2>0.9$). The relative contributions of PCBs 194, 201, and 206 to ΣPCB concentrations were greater in tissues than diet. Polychlorinated biphenyl 209 was also elevated in adult female adipose, but decreased in kit and juvenile adipose tissues, compared to diet (Figure 1).

Polychlorinated dibenzo-p-dioxins accounted for 64% ± 18 (mean ± SD) of the total ΣTEQ in Aroclor 1268 treatment diets, with PCBs accounting for the remaining 36% ± 18. The contribution of PCDFs to ΣTEQs was negligible (0.5%±0.3). 1,2,3,7,8-PeCDD accounted for 82% ± 2 of PCDD/PCDF ΣTEQs. Polychlorinated biphenyl-126 accounted for 96% ± 0.02 of non-ortho PCB ΣTEQs in Aroclor 1268 treatment diets and 36% ± 18 of total ΣTEQs. Sum TEQs in all Aroclor 1268 treatments were lower than the positive control. Polychlorinated biphenyl-126 accounted for 100% of ΣTEQs in the positive control, which measured 90.2 and 132 pg ΣTEQ/g feed for the first and second batches, respectively.

Lipid normalized diet-to-adipose biomagnification factors (BMFs) were greater for adult females than juvenile mink. The exposure period for kit mink was insufficient to reach equilibrium; hence, BMFs were not evaluated for kits. Diet-to-liver BMFs were greater than diet-to-adipose BMFs in both adult and juvenile mink (Figure 2).







*µg Σ[PCB]/g lipid / µg Σ[PCB]/g lipid

Figure 2 (a,b,c). Biomagnification factors and ratios of PCBs in tissues by treatment*

Adult female diet-to-adipose-BMFs ranged from 7.5 to 13 (mean=11), while they ranged from 2.6 to 4.1 in juvenile mink (Figure 2a). Adult female diet-to-liver BMFs ranged from 9.8 to 40 (mean=23) compared to a range of 4.5 to 10.8 in juvenile mink (Figure 2b). The relationship between BMFs and treatment in adult female mink was non-monotonic with the greatest BMFs observed in intermediate treatments and lesser values at the low and high ends of the dosing regime. This differed from juvenile mink, for which BMFs were negatively correlated with dietary ΣPCB concentration ($p<0.05$). The ratios of ΣPCB concentrations in liver compared to adipose were similar in both age classes, ranging from 1.6 to 4.7 in adult female mink, and 1.7 to 2.9 in juvenile mink (Figure 2c).

Gestation, whelp success, and litter size

Gestation duration for all mink ranged from 40 to 60 days with a median length of 47 days and did not correlate with ΣPCBs in diet or adipose ($p>0.05$). Forty of 43 dams exposed to Aroclor 1268 whelped kits, although two dams in the 17.1 µg ΣPCB/g feed group whelped stillborn litters. No significant differences ($p<0.05$) were observed in the percentages of females that whelped live litters in control and the PCB dietary treatment groups (Table 3).

Table 3. Reproductive measurements by treatment

	Gestation duration (days)		Females whelped live litters			Stillborn kits/ Total kits			Total kits per litter[a]		Live kits per litter[b]	
	n	Mean ± SD	n	Count	%	n	Count	%	n	Mean ± SD	n	Mean ± SD
Negative control	10	46 ± 1.6	10	10	100	61	5	8.2	10	6.1 ± 2.3	10	5.3 ± 2.4
Aroclor 1268 1	8	50 ± 4.6	9	8	89	42	1	2.4	8	5.3 ± 0.89	8	5.0 ± 1.1
Aroclor 1268 2	10	45 ± 2.8	10	10	100	57	1	1.8	10	5.7 ± 1.7	10	5.6 ± 1.8
Aroclor 1268 3	9	47 ± 5.1	10	9	90	35	1	2.9	9	3.9[d] ± 2.4	9	3.8 ± 2.2
Aroclor 1268 4	10	47 ± 1.9	10	8	80	34	4	12	10	3.4[d] ± 1.3	8	3.3[c] ± 1.7
Aroclor 1268 5	3	50 ± 7.6	4	3	75	16	4	25[d]	3	5.3 ± 3.2	3	4.0 ± 1.0
Positive control	7	45 ± 1.7	8	5	63	30	5	17	7	4.3 ± 1.3	5	3.6 ± 2.0

a. Includes stillborn kits

b. Excludes stillborn kits

c. $p<0.05$

d. $p<0.01$

A significant increase in the proportion of stillborn kits was observed in the 28.8 µg ΣPCB/g feed group ($p<0.01$), but not for other Aroclor 1268 treatments ($p>0.05$), when compared to the control group. A negative correlation was observed between live litter size and ΣPCB concentration in diet and liver ($p<0.05$), but not adipose ($p>0.05$), while the 17.1 µg ΣPCB/g feed group was the only treatment significantly less than the control ($p<0.05$). Gestation and litter size were unaffected in the positive control ($p>0.05$), while whelp success was less than the in control group (67%, $p<0.05$) (Table 3).

Body condition index and growth

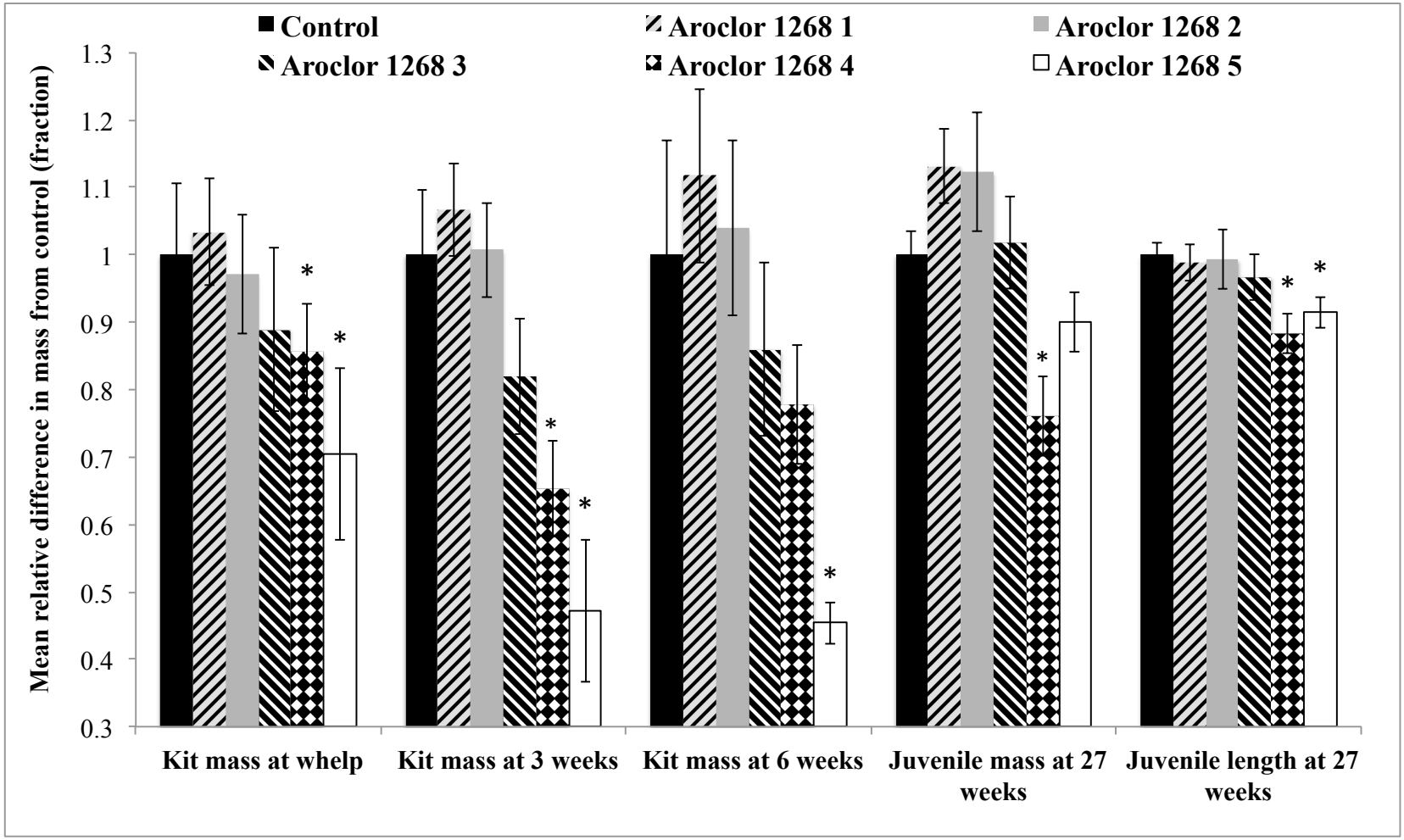
At the initiation of treatment diet feeding, neither adult female body masses nor BCI differed across treatments ($p>0.05$). Twenty-four hours after whelp, dam body mass and BCI correlated negatively with dietary ΣPCB concentration ($p<0.0001$ and <0.01, respectively). Mean treatment BCIs of dams in the 17.1 and 28.8 µg ΣPCB/g feed group 24 hours post whelp were less than control ($p<0.05$), consistent with the groups that displayed decreased feed consumption (Table 4).

Table 4. Mean body condition index by treatment

	Adult females at initiation		Adult females at whelp		Juveniles at 27 weeks	
	n	**Mean ± SD**	**n**	**Mean ± SD**	**n**	**Mean ± SD**
Negative control	10	14 ± 77	10	33 ± 79	9	-68 ± 90
Aroclor 1268 1	8	46 ± 110	8	78 ± 42	10	130 ± 110
Aroclor 1268 2	10	-22 ± 110	10	32 ± 95	10	-7.5 ± 110
Aroclor 1268 3	9	13 ± 160	9	-26 ± 85	9	23 ± 95
Aroclor 1268 4	10	-28 ± 69	10	-71* ± 97	10	-97 ± 180
Aroclor 1268 5	3	-34 ± 150	3	-110* ± 85	2	69 ± 32
Positive control	7	-4.0 ± 180	7	36 ± 114	9	-16 ± 120

* $p<0.05$

Mean live kit mass at whelp was less than the control in the 17.1 and 28.8 µg ΣPCB/g feed groups ($p<0.05$ and <0.001, respectively). Mean kit mass at 3 weeks of age was less than control in the 10.6, 17.1, and 28.8 µg ΣPCB/g feed groups ($p<0.05$, <0.0001, and <0.01, respectively). At 6 weeks of age, the 28.8 µg ΣPCB/g feed treatment was the only group with mean kit mass less than the control ($p<0.05$). Mean kit mass in the positive control was less than the control at whelp, 3 weeks, and 6 weeks of age ($p<0.05$) (Figure 3).



*p<0.05

Figure 3. Relative difference from the control of growth measurements for mink from whelp to 27 weeks

Juvenile mink body masses at 27 weeks of age correlated negatively with ΣPCB concentration in diet and adipose ($p<0.05$). The only treatment for which mean juvenile mink body mass of juvenile mink that was significantly less than control was the 16.8 µg ΣPCB/g feed group ($p<0.05$). However, the statistical comparisons for the 28.8 µg ΣPCB/g feed group had low power, due to kit mortality and associated low juvenile sample size (n=2). Consistent with the body mass results, juvenile mink body length at 27 weeks correlated negatively with ΣPCB concentration in diet ($p<0.01$). Body lengths of juvenile mink in the 16.8 µg ΣPCB/g feed group were less than control ($p<0.01$) (Figure 3). Juvenile mink BCI at 27 weeks correlated negatively with ΣPCB concentrations in adipose ($p<0.05$), but not in diet or liver ($p>0.05$), and differences between treatments were not significant. Correlations between body mass, body length, and BCI with PCB exposure were not significant when the 16.8 and 28.8 µg ΣPCB/g feed groups were excluded ($p>0.05$). Juvenile mink growth was not significantly affected by the positive control ($p>0.05$) (Figure 3, Table 4).

Mortality

Kit lifespan from whelp to 6 weeks was negatively associated with dietary ΣPCB concentration ($p<0.01$). Except for the 4.01 µg ΣPCB/g feed group, the percent of mortality from infanticide was greater in all Aroclor 1268 treatment groups compared to control ($p<0.0001$). In the control group, only one out of 61 kits died from infanticide (Table 5).

Table 5. Kit mortality and infanticide from whelp to 6-weeks of age

	Total kit mortality			Litters with infanticide			Infanticide/ total live kits			Infanticide/ total kit mortality		
	n	Count	%	n	Count	%	n	Count	%	n	Count	%
Negative control	61	6	10	10	1	10	61	1	1.6	6	1	17
Aroclor 1268 1	42	13	31	8	3	38	42	9	21	13	9	69
Aroclor 1268 2	57	4	7.0	10	0	0	57	0	0	4	0	0
Aroclor 1268 3	35	6	17	9	1	11	35	4	11	6	4	67
Aroclor 1268 4	34	16	47	8	5	63	34	12	35	16	12	75
Aroclor 1268 5	16	14	88	3	1	33	16	1	6.3	14	1	7.1
All Aroclor 1268 treatments	184	53	29	38	10	26	184	26	14	53	26	49
Positive control	30	9	30	7	2	29	30	5	17	9	5	56

To account for kit mortality from infanticide, data were censored prior to a survival analysis. When infanticide was censored, the 28.8 μg ΣPCB/g feed treatment was the only group with mean kit lifespan that was less than that of the control ($p<0.0001$). When infanticide was included in survival analysis, lifespans of kits in the 1.77, 17.1, and 28.8 μg ΣPCB/g feed groups and the positive control were less than the control for total kits whelped ($p<0.05$, <0.001, <0.0001, and <0.05, respectively) and live born kits ($p<0.05$, <0.01, <0.0001, and <0.05, respectively). Lifespans of total kits whelped and live born kits in the 4.01 and 10.6 μg ΣPCB/g feed groups were not different from the control when infanticide is included in survival analyses ($p>0.05$).

Toxicity benchmark estimates

No and lowest observed adverse effect concentrations (NOAECs/LOAECs) were identified, as well as effect and lethal concentration estimates to describe toxicity benchmarks for selected endpoints. Diet and tissue based NOAECs and LOAECs for reproductive, growth, and mortality endpoints are summarized in Table 6.

Table 6. Diet and tissue based NOAECs and LOAECs for reproductive, growth, and mortality endpoints

Reproductive Endpoints	NOAEC				LOAEC			
	Diet		Adipose[a]	Liver[a]	Diet		Adipose[a]	Liver[a]
	µg ΣPCB/g ww	pg ΣTEQ/g ww	µg ΣPCB/g lipid	µg ΣPCB/g lipid	µg ΣPCB/g ww	pg ΣTEQ/g ww	µg ΣPCB/g lipid	µg ΣPCB/g lipid
Litter size[b]	4.01	7.69	492	532	10.6	35.1	1330	4100
Live litter size[b]	10.6	35.1	1330	4100	17.1	52.2[c]	2350	4690
Proportion of kits stillborn	17.1	52.2[c]	2350	4690	28.8	85.1	2110	6330
Kit mortality whelp to 6 weeks w/ infanticide	10.6	35.1	649	4100	17.1	52.2[c]	2350	4690
Kit mortality whelp to 6 weeks w/o infanticide	17.1	52.2[c]	2350	4690	28.8	85.1	2110	6330
Growth Endpoints								
Adult body condition index (BCI)	10.6	35.1	1330	4100	17.1	52.2[c]	2350	4690
Kit mass at whelp[b]	10.6	35.1	1330	4100	17.1	52.2[c]	2350	4690
Kit mass at 3 weeks[b]	4.01	7.69	492	532	10.6	35.1	1330	4100
Kit mass at 6 weeks[b]	17.1	52.2[c]	2350	4690	28.8	85.1	2110	6330
Juvenile body mass and length at 27 weeks	8.1	12.3[c]	266	579	16.8	51.4[c]	526	1080

[a] All values reflect treatment mean PCB concentrations of adult female mink with the exception of juvenile body length and mass
[b] 28.8 µg ΣPCB/g feed group excluded
[c] Value estimated from dietary ΣTEQ vs ΣPCB standard curve (Supplemental Figure 1)

Various control adjusted EC (or LC) values were estimated from dose-response curves for kit mass at 6 weeks, juvenile length at 6 weeks, and kit mortality at 6 weeks in terms of diet, liver, and adipose ΣPCB concentrations (Figures 4, 5, 6, 7, 8, 9, A2, A3, A4, A5, A6, A7, and Table 7).

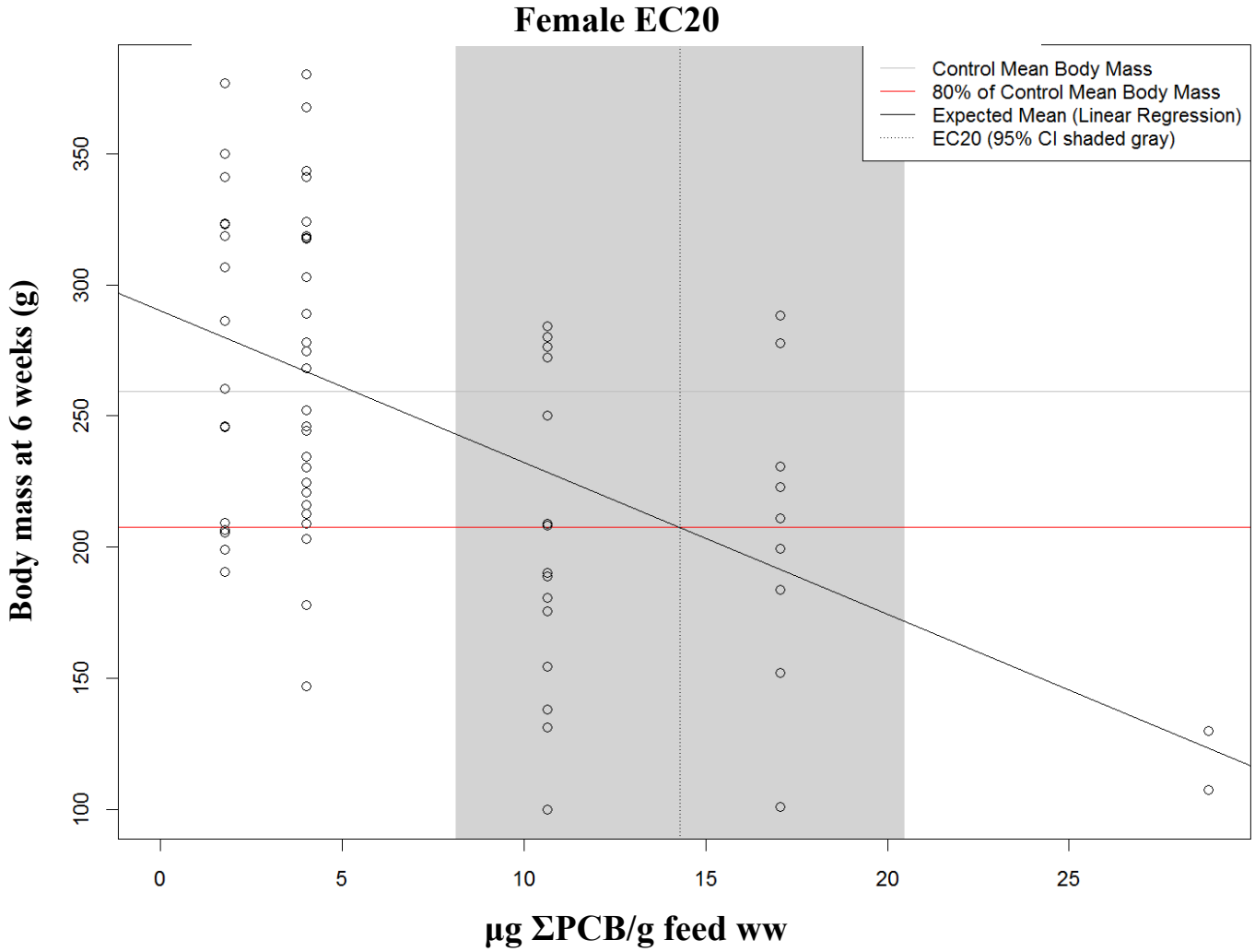


Figure 4. Dose-response and diet PCB EC20 benchmark value for female kit body mass at 6 weeks

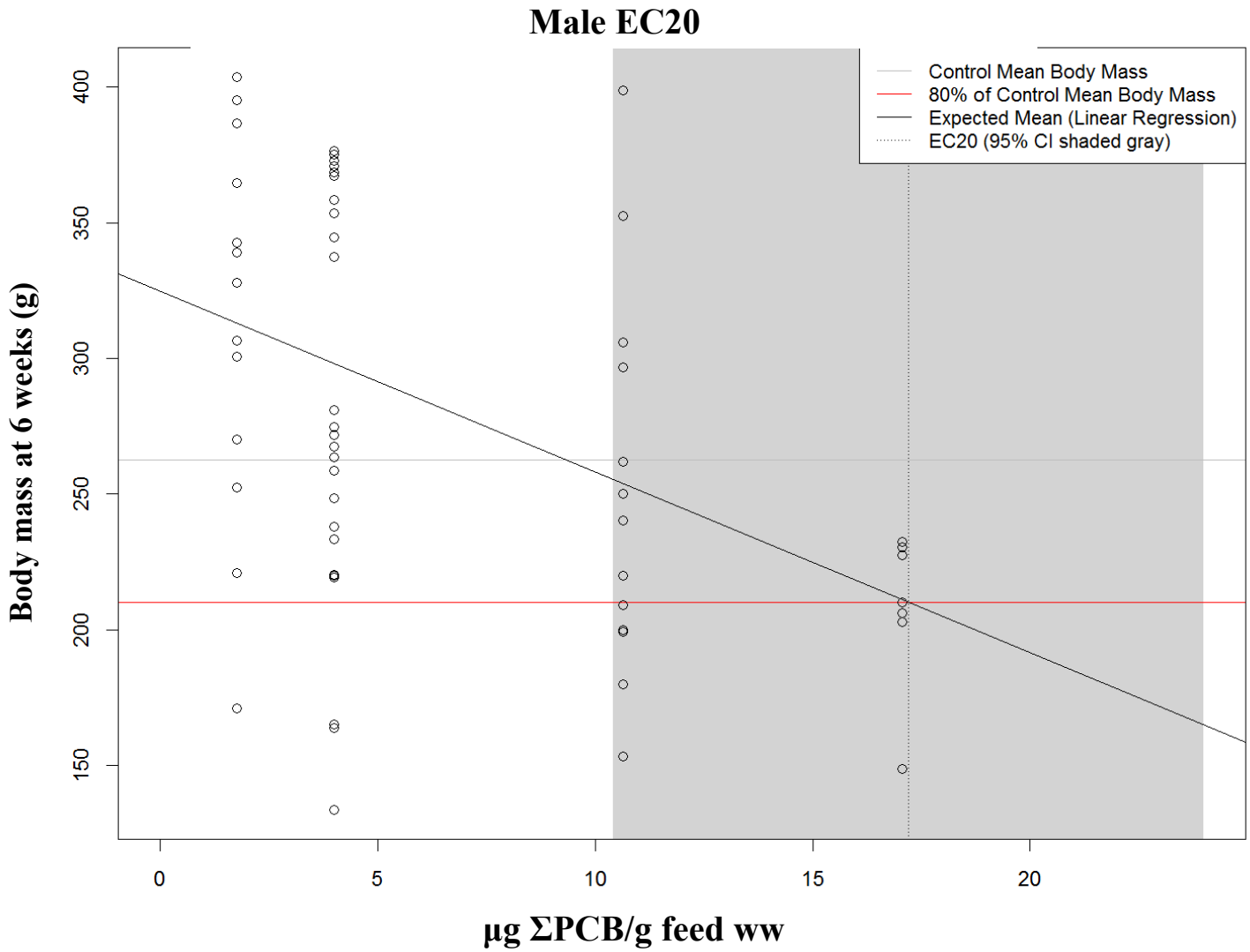


Figure 5. Dose-response and diet PCB EC20 benchmark value for male kit body mass at 6 weeks

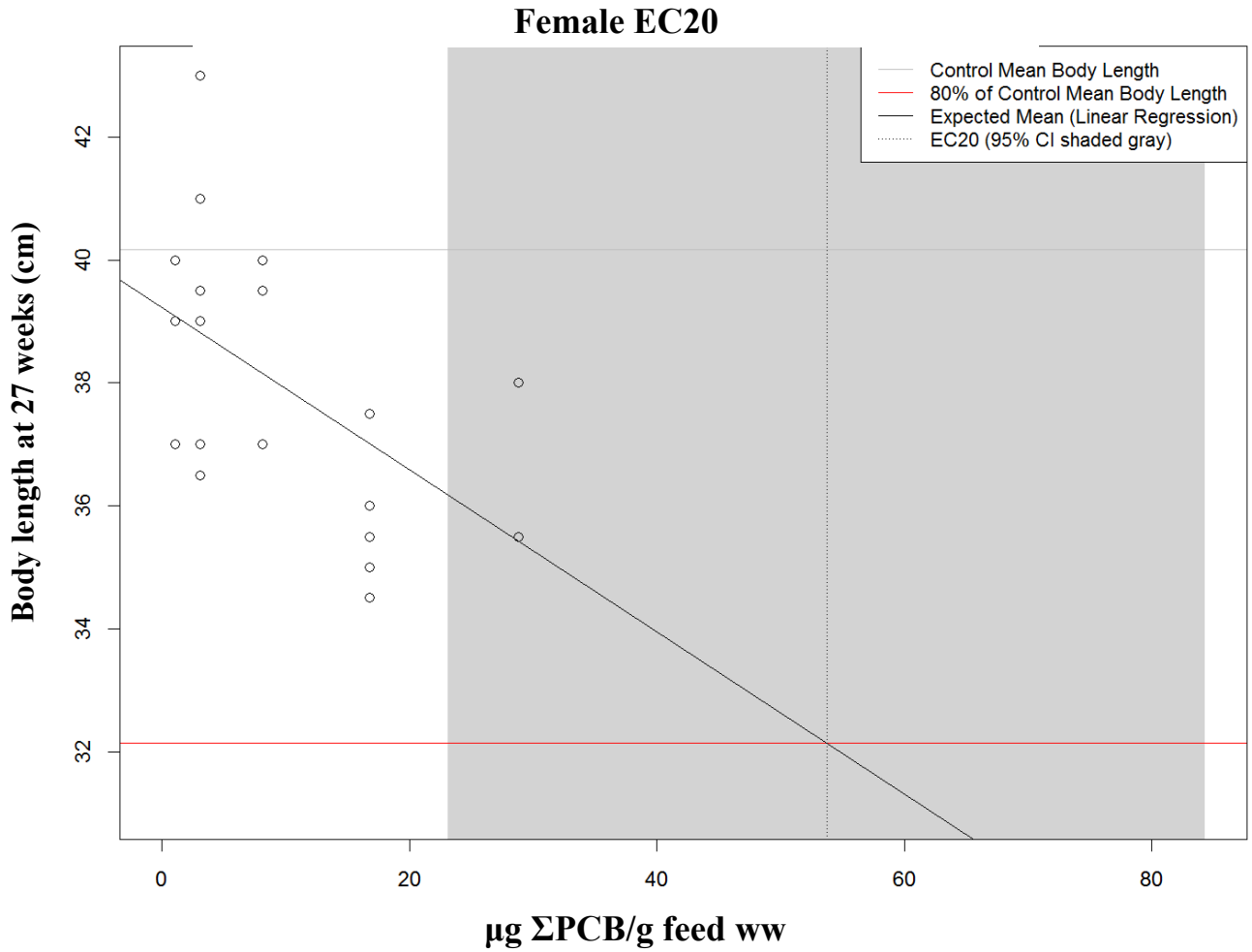


Figure 6. Dose-response and diet PCB EC20 benchmark value for female juvenile body length at 27 weeks

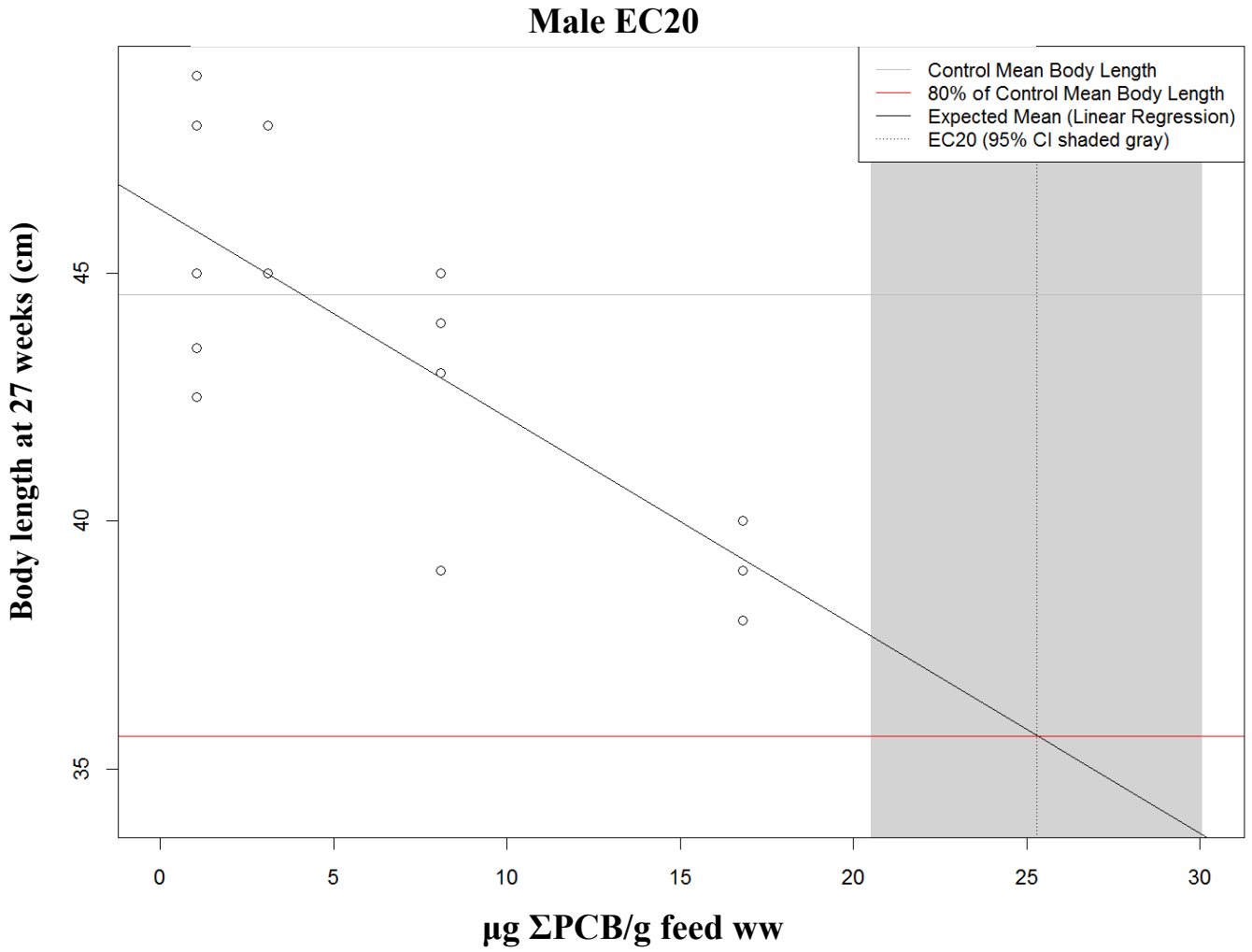


Figure 7. Dose-response and diet PCB EC20 benchmark value for male juvenile body length at 27 weeks

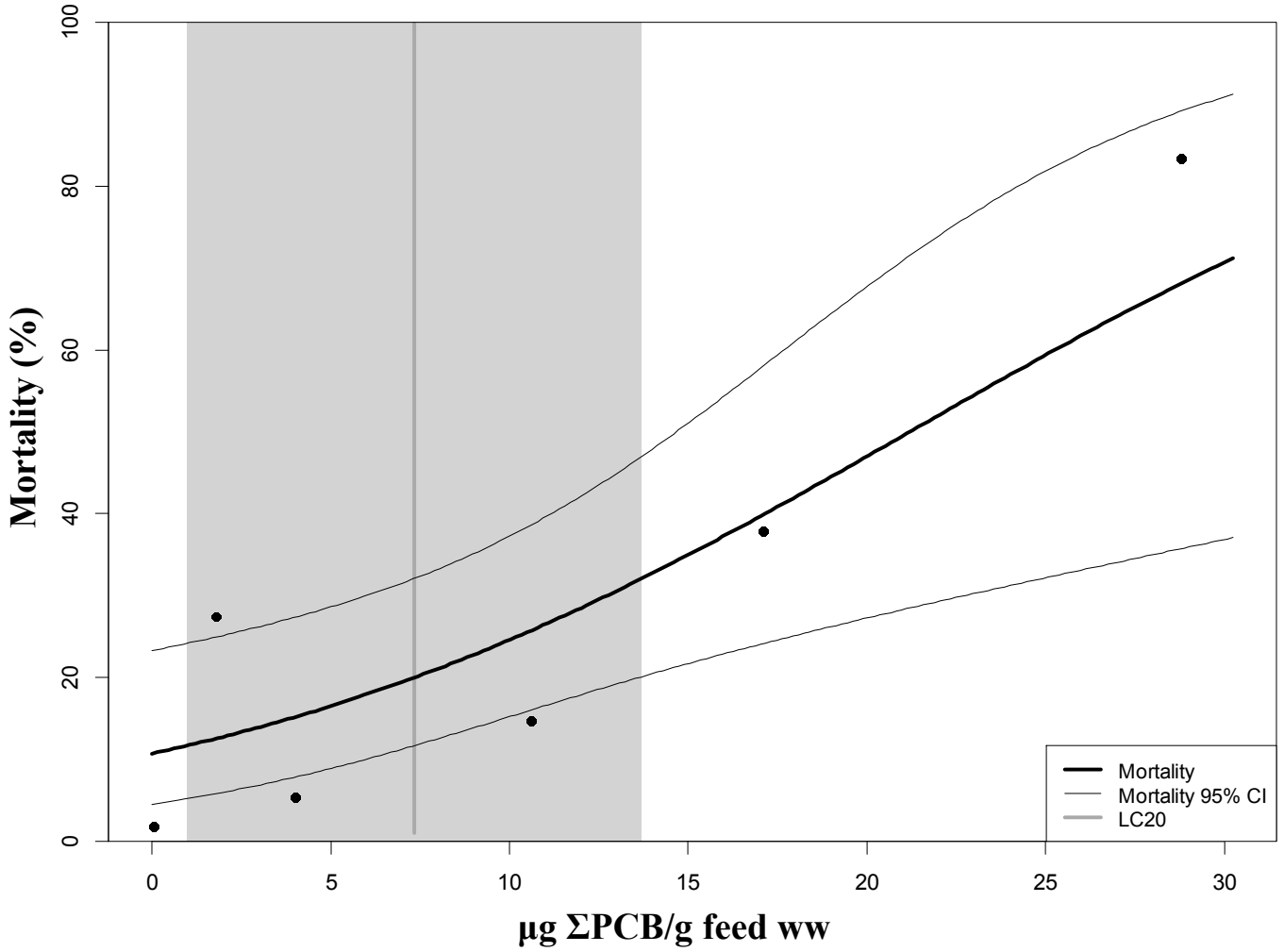


Figure 8. Dose-response and diet PCB LC20 benchmark value for kit mortality at 6 weeks

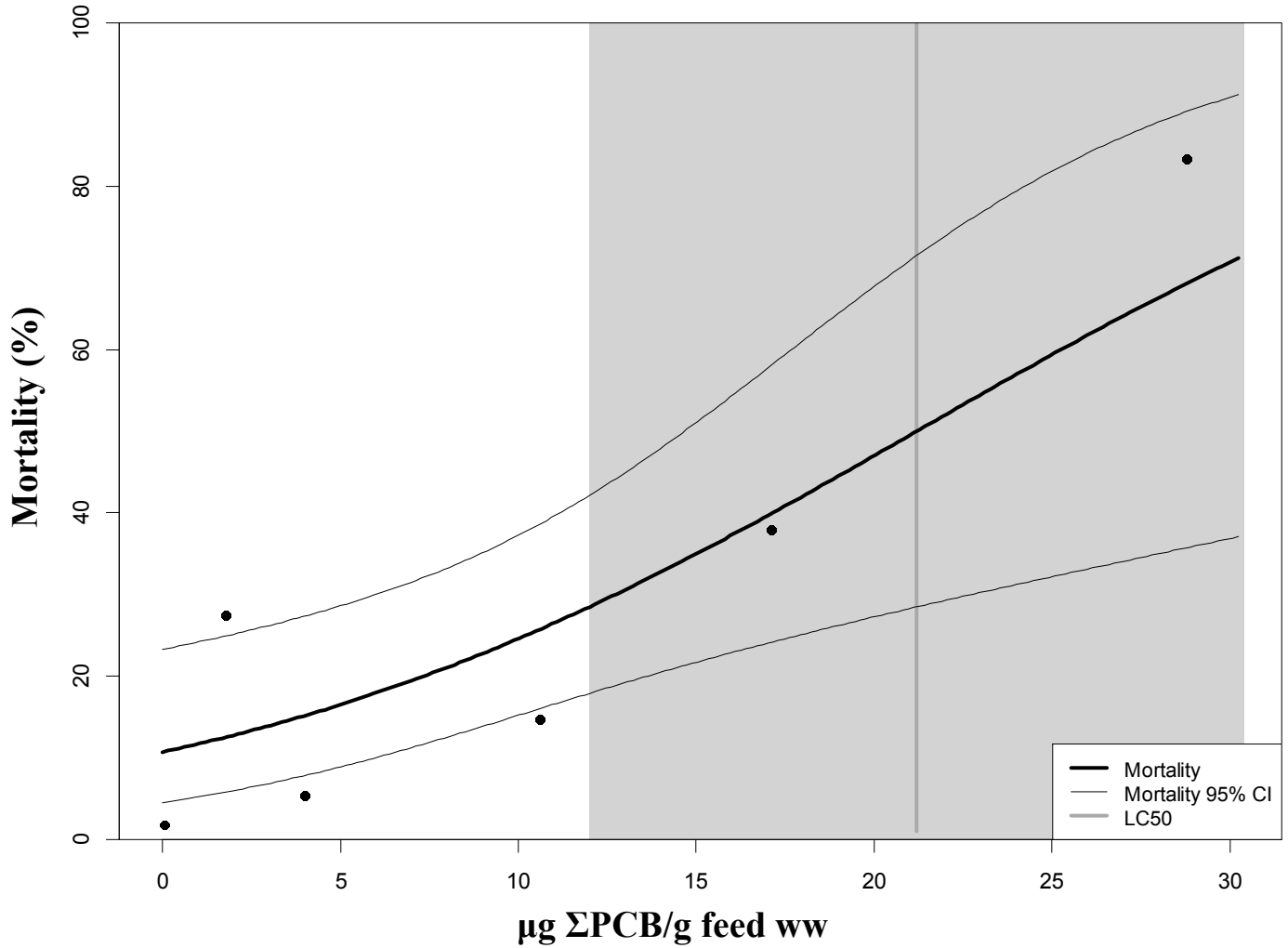


Figure 9. Dose-response and diet PCB LC50 benchmark value for kit mortality at 6 weeks

Table 7. Diet, liver, and adipose 20 and 50% EC/LC estimates for kit mass and mortality at 6 weeks of age and juvenile length at 27 weeks of age

		6 week kit mass						6 week kit mortality[ab]					
			95% CI			95% CI			95% CI			95% CI	
Exposure metric[c]	Sex	EC20	LCL	UCL	EC50	LCL	UCL	LC20	LCL	UCL	LC50	LCL	UCL
Diet	Male	17	10	24	29	17	41	7.34	0.975	13.7	21.2	12.0	30.4
	Female	14	8.1	21	28	20	35						
Maternal liver	Male	188	98	278	329	165	494	148	135	244	307	135	479
	Female	146	79	212	291	208	374						
Maternal adipose	Male	1470	916	2020	2570	1660	3480	933	220	1770	2170	489	3860
	Female	1120	479	1750	2320	1220	3410						

		27 week juvenile length					
			95% CI			95% CI	
	Sex	LC20	LCL	UCL	LC50	LCL	UCL
Diet	Male	25	21	30	57	43	72
	Female	54	23	84	145	54	236
Juvenile liver	Male	86	66	106	191	134	247
	Female	107	67	146	274	152	396
Juvenile adipose	Male	727	598	856	1640	1250	2030
	Female	1300	774	1840	3460	1830	6000

[a]Includes infanticide
[b]Sexes combined
[c]µg ΣPCB/g ww
UCL: 95% upper confidence limit
LCL: 95% lower confidence limit
EC: Effect concentration
LC: Lethal concentration

DISCUSSION

Bioaccumulation

It was uncertain whether the administered dietary concentrations of Aroclor 1268 would achieve adipose concentrations in adult mink that were comparable to those in TBRE bottlenose dolphin blubber, especially because the treatment concentrations had to be reduced due to poor palatability. Evidence suggested that Aroclor 1268 might not partition in to tissues efficiently due to steric factors and the extremely hydrophobic nature of the congeners in the mixture (Kannan et al. 1998). While biomagnification factors generally increase with lipophilicity (expressed as the octanol-water partitioning coefficient, log K_{ow}), studies have shown that this relationship may plateau as log K_{ow} values approach or exceed 7.5 (Hornshaw et al. 1983, Tillitt et al. 1996, Maruya and Lee 1998,). The most abundant PCB congeners in Aroclor 1268 have average log K_{ow} values near 7.9 (Kannan et al. 1998, Kannan 1999). During planning of this study, it was hypothesized that partitioning of Aroclor 1268 could be limited by the extreme hydrophobicity of the mixture, but that the capacity of mink (and cetaceans) to biotransform and eliminate PCBs is generally limited to lower chlorinated congeners, and the highly chlorinated PCBs would undergo little metabolism and rapidly accumulate once assimilated into tissues (Tanabe et al. 1988, Leonards et al. 1998). To ensure PCB tissue concentrations relevant to TBRE dolphins were reached, the concentrations of the Aroclor 1268 in treatment diets were greater than most previous laboratory mink studies that used other Aroclors and environmental mixtures (Fuchsman et al. 2008 and references therein, Bursian et al. 2013).

The dietary PCB concentrations administered were intended to reflect those in preferred prey species of TBRE bottlenose dolphins and result in PCB concentrations in mink adipose that would bracket PCB concentrations in reported in TBRE dolphin blubber. Concentrations of PCBs in dolphin prey species collected from the TBRE have been reported for striped mullet, spot, silver perch, and spotted sea trout (113 ± 25.7, 83.1 ± 60.6, 41.3 ± 11.3, and 8.21 ± 19.8 μg/g lipid, respectively, (mean ± SD)) (Pulster et al. 2005). The lowest three Aroclor 1268 treatment diets administered to adult female and kit mink (means=157, 492, and 1330 μg ΣPCB/g lipid, respectively) bracket these concentrations and the highest two treatments exceeded them (means=1610 and 1680 μg ΣPCB/g lipid, respectively). These highest two Aroclor 1268 treatments (17.1 and 28.8 μg ΣPCB/g feed) were predicted to result in adipose PCB concentrations in mink that were similar to PCB concentrations in blubber of breeding female TBRE dolphins (Tables 1 and 2). In actuality, ΣPCB lipid normalized BMFs were similar or greater than those documented in mink exposed to lesser chlorinated PCB mixtures (diet-to-adipose ~20, diet-to-liver ~3); hence, the extreme hydrophobicity of the PCB congeners characteristic of Aroclor 1268 did not effectively limit partitioning to the extent expected. Adipose PCB concentrations in mink relevant to TBRE dolphins were achieved and even exceeded. In fact, ΣPCB concentrations in adipose tissues of adult female mink in this study were greater than any reported in PCB mink feeding studies of similar design. Sum PCB concentrations in adipose tissue of adult female mink fed 1.77 μg ΣPCB/g feed (157 ± 73.5 μg ΣPCB/g lipid, (mean ± SD)) bracketed the mean and 95% upper CI ΣPCB concentrations reported in female TBRE bottlenose dolphin blubber (117 and 174 μg ΣPCB/g lipid, respectively) and the 10.6 μg ΣPCB/g feed group adipose ΣPCB

concentrations (1330 ± 981 µg ΣPCB/g lipid) bracketed similar values for male TBRE dolphins (510 and 704 µg ΣPCB/g lipid, respectively; Balmer et al. 2011). The highest adipose ΣPCB concentrations were in mink in the 28.8 µg ΣPCB/g feed treatment group and reached 2850 µg ΣPCB/g lipid in a post lactation adult female and 4730 µg ΣPCB/g lipid in an adult female that did not produce a litter. These values exceed the greatest PCB concentration reported in adipose tissue of any post-lactation female TBRE bottlenose dolphin (338 µg ΣPCB/g lipid) and rival the highest concentration reported in males (2900 µg ΣPCB/g lipid) even when species differences in lipid content of adipose tissue are considered (65% for adult female mink vs. 33% for female TBRE dolphins and 25% for male dolphins (Balmer at al. 2011 and Kucklick et al. 2011)).

Measurements of hepatic PCB concentrations for TBRE dolphins are scarce; thus, comparisons to hepatic PCB concentrations in mink are not feasible. However, these measurements are useful for describing the bioaccumulation and toxicokinetics of Aroclor 1268 that likely also apply to marine mammals. Polychlorinated biphenyl 194, 201, 206, and 209 had greater relative abundances in adipose and hepatic tissues than diet. These 4 congeners contributed >75% of total PCB concentrations in tissues, while the relative abundances of the other seven Aroclor 1268 congeners were greater in diet as compared to tissues (Figure 1). The congeners 194, 201, 206, and 209 were also differentially retained in the livers of both adult and juvenile mink, which had ΣPCB concentrations 2.3 to 2.6 times greater than in adipose (Figure 2). Hepatic sequestration is the mechanism by which PCDDs, PCDFs, and dioxin-like PCBs preferentially accumulate in the liver following binding to CYP450 enzymes abundant in the microsomal fraction of hepatocytes. However, the 4 PCBs that preferentially

accumulated in livers of Aroclor 1268 treated mink in this study have 3 or more ortho-substituted chlorines and little to no binding affinity for CYP450 enzymes due to steric hindrance. Hence, typical hepatic PCB sequestration driven by irreversible binding to CYP450 enzymes does not explain the observed preferential accumulation of PCBs 194, 201, 206, and 209 (Giesy and Kannan 1998, Ngui and Bandiera 1999). Lipid composition differences can also affect the relative accumulation of PCBs in tissues. The lipid composition of liver is distributed between phospholipids, non-esterified fatty acids, and triglycerides, while adipose tissue is dominated by triglycerides (Tan et al. 1984, Kawai et al. 1988). A greater affinity of phospholipid membranes for PCB 194, 201, 206, and 209 could also provide a possible explanation for the preferential hepatic accumulation of PCBs observed in mink here (Endo et al. 2011).

Biomagnification factors and ΣPCB concentrations in adipose and livers of adult female mink were greater than juvenile mink despite a shorter exposure period and the benefit of PCB offloading through lactation. Also recall that diet-to-liver BMFs in adult female mink were non-linear with respect to dietary PCB exposure, while BMFs decreased with dietary ΣPCB concentration in juvenile mink (Figure 2). Growth dilution and the slightly lower ΣPCB concentrations in Aroclor 1268 diets administered to juveniles when compared to diets given to adults and kits may account for some of the observed differences in accumulation. In addition, the lipid content of adipose tissue of adult female mink differed from juvenile mink (64% in adults, 90% in juveniles) (Figure 2); hence lipid normalizing has a greater effect on adult female adipose PCB concentration than juvenile mink. Furthermore, periods of high energetic demand (i.e. reproduction) and resource scarcity (mimicked here by the feed palatability issue) have

the potential to change lipid content of adipose tissue and bioamplify organohalogen concentrations in tissues of mammals when weight loss exceeds the rate of elimination (Nakata et al. 1998, Lydersen et al. 2002, Daley et al. 2014).

Feed Consumption

The poor palatability of feed in the greater PCB treatment groups was a negative consequence of the goal to achieve PCB concentrations in mink greater or equal to those noted in TBRE bottlenose dolphins. To our knowledge, this is the first time poor palatability has been observed with a commercial PCB mixture, which are widely considered to be tasteless and odorless. Treatment diets containing ΣPCB concentrations greater than 28.8 µg ΣPCB/g feed were originally proposed, but were either refused or consumed at a rate that would not sustain adult female mink. No gastrointestinal distress, vomiting, or any other signs of illness were observed. Dilution of refused Aroclor diets with control diet reversed the refusal, and re-mixing of the high-dose PCB diets with the same ingredients did not affect consumption. Each of these manipulations, as well as the instantaneous refusal for diets greater than 28.8 µg ΣPCB/g feed, is consistent with decreased feed consumption related to palatability and not with wasting syndrome. Initially, the 17.1 and 28.8 µg ΣPCB/g feed diets appeared to be the highest concentrations for which mink would consume an entire daily allotment. However, between initiation of the exposure period and whelping, reduced feed consumption became obvious, in conjunction with diminished nutritional status. A noteworthy treatment effect itself, decreased feed consumption is a confounding variable when attributing adverse effects observed in mink to either PCB-related toxicity

and/or changes in nutritional status. This is a critical consideration when interpreting the results of this study. Any endpoint assessed, particularly those associated with growth, that had dietary lowest observed adverse effect concentrations (LOAECs) of 17.1 and 28.8 µg ΣPCB/g feed were likely influenced by nutritional state, but the extent of that influence is unknown. Pronounced treatment-related differences in feed consumption occurred in adult female mink, but not in juvenile mink, where Aroclor 1268 treatment BCIs were not different from the control values. Additionally, no obvious decrease in feed consumption by juvenile mink was noted in the study, unlike that observed in adult females. One hypothesis for this observation is that the F1 generation may have been conditioned to the flavor of treatment feed since it had been consumed from birth unlike the adult female mink. This also supports the hypothesis that the issue was indeed palatability, and it did not affect the F1 generation to the extent that it did adult female mink.

Reproduction, growth, and mortality

Endpoint effects in positive control mink provide evidence that study animals were indeed sensitive to PCBs to the extent expected. Significant effects on reproduction and growth were not observed in the positive control relative to the negative control, although some dams exposed to the positive control gave birth to stillborn litters, which was expected since the concentration of the positive control (~1 ng PCB 126/g feed ww) falls between the previous reproductive dietary based no observed adverse effect concentration (NOAEC) and lowest observed adverse effect concentration (LOAEC) for PCB 126 reported in Beckett et al. (2008). Histopathology of

juvenile mink jaws revealed a 100% incidence of severe squamous epithelial proliferations, which supports the assumption that study mink were characteristically sensitive to PCB exposure (Folland et al. 2015) given that the exposure concentration was >10x the PCB 126 level previously demonstrated to cause severe jaw lesions (Render et al. 2000).

Many effects of PCB exposure in this study were non-specific and are similar to those associated with diminished nutritional status, such as impaired reproductive and physiological function. The way mink were housed and fed was not conducive to the collection of reliable, individually-based feed consumption measurements because feed was lost through the bottom of the elevated cages, and mink also cached feed in nest boxes. A tool was needed to help distinguish effects from the related covariates of dietary PCB concentration and nutritional status that did not require precise measurements of feed consumption. Various forms of BCIs have been used as surrogate metrics for nutrition and individual health (Dobson 1992, Dobson and Michener 1995, Bachman and Widemo 1999, Miller and Hickling 1990). However, the validity of BCIs as indicators of individual health has been a subject of debate (Green 2001, Peig and Green 2009). While BCIs do not directly measure body fat reservoirs or nutritional condition, body condition index can be a reliable surrogate for these metrics when used as an index that compares fully developed animals of similar sex and age. Body condition index was the best available tool for this purpose. The ordinary least squares regression method that uses mass-to-size residuals to calculate BCI has been validated as an index of body condition for mammals (see methods, Schulte-Hostedde et al. 2001, 2005). Because dietary PCB concentration and feed palatability were

dependent, collinear variables, treatment mean and individual BCI values must be considered for statistically significant endpoints to be appropriately interpreted.

Polychlorinated biphenyl exposures did not cause overt reproductive failure in adult female mink at the concentrations tested despite those concentrations being notably high. Furthermore, reproductive and growth endpoints in the 1.77 and 4.01 μg ΣPCB/g feed treatments were not adversely affected relative to the control. The percent of females in Aroclor 1268 treatments that whelped litters with at least one live kit were not different from the control, with only three failing to whelp kits. The female mink that did not whelp litters were distributed among the 1.77, 10.6, and 28.8 μg ΣPCB/g feed Aroclor 1268 treatments. Placental reabsorption, if observed, could have been an indicator of PCB induced reproductive failure. However, the uteruses of Aroclor 1268 treated mink that failed to whelp in this study lacked characteristic placental scarring, indicating that either insemination or embryonic implantation had not occurred. Some reproductive measurements were adversely affected by Aroclor 1268 exposure. Total litter size, litter size of live kits, and live kit masses at whelp were affected by treatment, having both treatment differences from the control and significant regressions with diet and adipose PCB concentration. Aroclor 1268 exposure was also related to decreased growth in the F1 generation. Growth endpoints were also adversely affected by Aroclor 1268 treatments. Kit masses at whelp, 3-weeks, and 6-weeks were less than control for varying treatments, and the body lengths of juvenile mink were negatively affected by treatment (Figure 3, Table 6). The dose-responses of kit body mass and juvenile body length versus diet PCB concentration demonstrate the relationship between impaired growth and exposure to Aroclor 1268 treatments (Figures 4, 5, 6, and 7; and Table 7),

which are supported by tissue based exposure measurements albeit with less predictive ability (Figures A2, A3, A4, and A5).

Reproductive and growth effects in this study cannot be attributed solely to PCB toxicity. The nutritional state of dams during critical phases of development like gestation and lactation likely also contributed to observed effects. Body condition index of the respective dams correlated significantly with all reproductive and growth endpoints. Most of these effects were first observed in the 17.1 and 28.8 μg ΣPCB/g feed treatments, which had lower mean BCI values than the control. Interpretation of the adversely affected reproductive and growth endpoints should include consideration of the nutritional issues encountered in this study.

Kit mortality endpoints were confounded by infanticide in addition to nutritional state of dams. Infanticide was defined by the disappearance of kits or the recovery of partially consumed carcasses. The 1.77, and 17.1 μg ΣPCB/g feed groups, as well as the positive control, clearly had greater infanticide rates than are typically observed in commercial mink farms and previous mink studies of similar design, indicating that nutritional state does not entirely explain the increased prevalence of infanticide. Average mortality rates of live born kits through weaning are typically near 20%, of which infanticide (or siblicide) accounts for 30%, or ~7% of live kits. The frequency of infanticide typically is greatest within the first two days after parturition and is rarely observed after one week of age (Wenzel et al. 1984, Martino and Villar 1990, Schneider and Hunter 1993), whereas infanticide in Aroclor 1268 exposed litters occurred throughout the 6-week period from whelp to weaning. Here, infanticide occurred in all treatments except 4.01 μg ΣPCB/g feed, including the positive control. Infanticide

accounted for the majority of kit mortality in all Aroclor 1268 treatments except for the 28.8 µg ΣPCB/g feed group (Table 5).

Although the cause of death of consumed kits was evidently attributed to infanticide by the dam, prior PCB induced mortality could not be entirely excluded since carcasses were not available to examine post-mortem. Consequently, mortality of kits from suspected infanticide had to be censored from the analysis to eliminate bias, which was done using the right-censored survival analysis technique (Clark et al. 2003). When infanticide is censored, the dietary LOAECs for stillbirth, live born kit lifespan, and lifespan of total whelped kits was 28.8 µg ΣPCB/g feed. For comparison, if infanticide is treated as a potentially toxic effect and included in mortality analyses, kit lifespan of the 1.77 µg ΣPCB/g feed group was less than the control. However, the statistical significance of this result was substantially driven by an entire litter that was consumed, and kit lifespans of the 4.01 and 10.6 µg ΣPCB/g feed groups were not less than the control, indicating that 17.1 µg ΣPCB/g feed is a more appropriate LOAEC for kit mortality that includes infanticide. However, the NOAEC/LOAEC method is inherently flawed in that it cannot provide uncertainty estimates. As such, LCp threshold estimates and their confidence intervals were estimated to provide more information about the lethality of Aroclor 1268 exposure, yielding LC20 and LC50 values of 7.34 and 21.2, respectively (Figures 8 and 9, and Table 7). The limitation of a NOAEC becomes apparent here where the LC20 estimate value is actually lower than the associated NOAEC (although within the 95%CI), demonstrating the principle that a NOAEC estimate is ultimately only one value in an unknown distribution of possible estimates. It should be noted that the beta-binomial method used for modeling kit mortality dose-

response does not include a method for censoring of infanticide. Hence, the dietary LC20 and LC50 values should be considered conservative threshold estimates for mortality because infanticide certainly influenced the analysis, although the extent of which is unknown.

Litter size and kit mass at 3 weeks were the most sensitive adversely affected endpoints assessed in this study, both having dietary LOAECs of 10.6 µg ΣPCB/g ww or 102 µg ΣPCB/g lipid (Table 6). This adverse effects threshold corresponded with treatment mean adipose and hepatic ΣPCB concentrations in adult female mink of 649 and 122 µg ΣPCB/g ww or 1330 and 4100 µg ΣPCB/g lipid, respectively. There were no adverse growth or reproductive effects in treatments equal to or less than 4.01 µg ΣPCB/g feed ww (40.3 µg ΣPCB/g lipid) for any endpoints assessed across all age classes. The corresponding adult female mean adipose and liver ΣPCB concentrations are 492 and 532 µg ΣPCB/g lipid, respectively (Table 6). In reviews of PCB effects on mink, Zwiernik et al. (2010) estimated a threshold for reproductive effects of PCBs as 41 µg/g lipid in mink liver, while Fuchsman et al. (2008) estimated a whole-body threshold equivalent to approximately 10 µg/g lipid. Also recall the reproductive TRVs provided in Kannan et al. (2000) of 17 µg ΣPCB/g lipid in blubber, and the EC10 of 14.8 µg ΣPCB/g lipid in blubber or liver provided by Schwacke et al. (2002). On this basis, reproductive effect thresholds for Aroclor 1268 in mink tissue observed here are one to two orders of magnitude higher than the environmental PCB mixtures considered in the development of previous TRVs.

Considering the above, it seems reasonable that previous TRVs would overestimate risk from Aroclor 1268 exposure to TBRE bottlenose dolphins. It should

be noted that application of these findings to dolphin exposures should consider factors such as interspecies differences in lipid composition, exposure scenario, and toxicokinetics that influence relative exposure and species sensitivity. The percent and composition of lipid in adipose tissue of mink versus blubber of dolphins, coupled with bioamplification of PCBs in mink that experienced weight loss might overestimate exposure in mink compared to an analogous concentration in bottlenose dolphin tissue. Conversely, the continuous exposure experienced by mink is akin to a hypothetical, unlikely scenario where TBRE dolphins consume forage solely from the contaminated site. While these differences influence interspecies extrapolation, the magnitude of PCB concentrations in tissues of mink that were associated with reproductive failure for Aroclor 1268 were remarkably high relative to threshold concentrations available for most Aroclors and environmental mixtures (Heaton et al. 1995, Brunström et al. 2001, Bursian et al. 2006ab Fuchsman et al. 2008, Zwiernik et al. 2009, Bursian et al. 2013). The fact that these PCB concentrations represent mink following the offloading PCBs via lactation adds to the significance of these results. With these considerations in mind, this study provides novel data to aid in the understanding kinetics, trophic transfer, and reproductive toxicity thresholds for PCB congeners characteristic of Aroclor 1268 to mink, TBRE bottlenose dolphins and aquatic mammals in general.

APPENDIX

Table A1. Polychlorinated biphenyl, polychlorinated dibenzo-p-dioxin, and polychlorinated dibenzo furan congeners targeted for analytical chemistry

Aroclor 1268 congeners by GC-ECD (EPA METHOD 8082)

Congener #	MDL (ng/mL)
PCB #180	6.08[a]
PCB #187	2.82
PCB #194	2.13
PCB #196	2.61
PCB #200	2.99
PCB #201	1.15[b]
PCB #202	0.99[c]
PCB #206	2.6
PCB #207	0.94[d]
PCB #208	2.47
PCB #209	2.45

*Congeners with similar letters coeluted

DL-PCBs by GC-HRMS (EPA METHOD 1668)

Congener #	MDL (ng/mL)
PCB #77	0.002
PCB #81	0.002
PCB #126	0.008
PCB #169	0.002

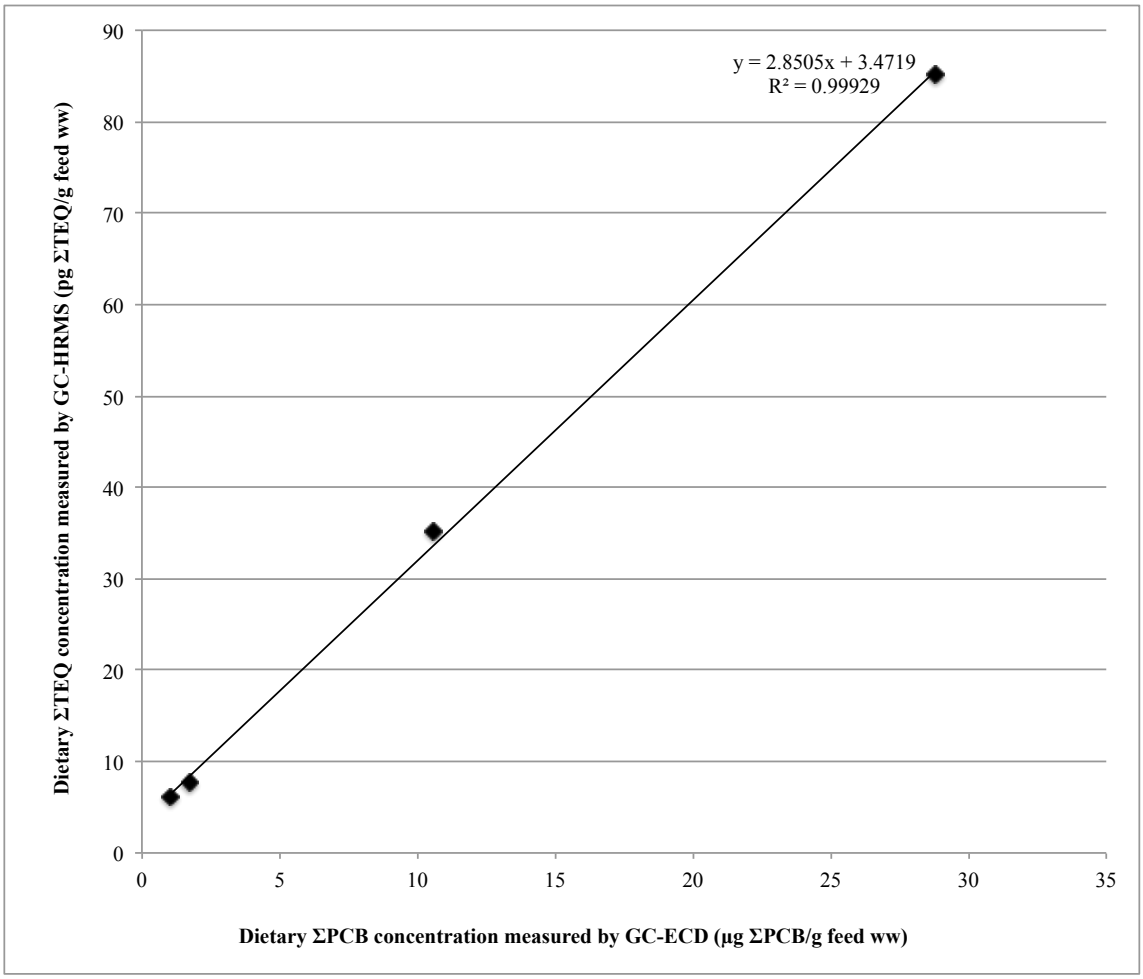
Additional congeners by GC-ECD (EPA METHOD 8082)

Congener #	MDL (ng/mL)	Congener #	MDL (ng/mL)
PCB #18	2.17	PCB #138	4.76[i]
PCB #28	2.24	PCB #146	1.15[f]
PCB #31	0.33	PCB #149	1.93
PCB #44	2.18	PCB #151	2.16
PCB #49	1.68	PCB #153	2.41[h]
PCB #52	2.08	PCB #154	1.31
PCB #56	2.58	PCB #156	3.08
PCB #66	2.51	PCB #157	0.05[j]
PCB #70	2.64	PCB #158	3.00
PCB #74	1.39[e]	PCB #163	4.76[i]
PCB #87	2.21	PCB #167	0.99[c]
PCB #92	2.08	PCB #170	1.15[b]
PCB #95	1.39[e]	PCB #172	0.05[j]
PCB #99	2.18	PCB #174	2.20
PCB #101	2.04	PCB #176	2.44
PCB #105	3.10	PCB #177	2.99
PCB #110	1.81	PCB #178	4.76[i]
PCB #118	2.55	PCB #183	3.07
PCB #119	2.20	PCB #185	0.77[g]
PCB #122	1.15[f]	PCB #189	0.94[d]
PCB #126	2.84	PCB #193	6.08[a]
PCB #128	0.77[g]	PCB #195	2.41
PCB #130	1.04	PCB #197	2.92
PCB #132	2.41[h]	PCB #199	6.08[a]
PCB #137	2.52	PCB #203	2.55

GC-HRMS PCDD and PCDF CONGENERS (EPA METHOD 1613)

Congener	MDL (ng/mL)
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)	0.008
1,2,3,7,8-Pentachlorodibenzo-p-dioxin (PeCDD)	0.002
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin (HxCDD)[K]	0.002
1,2,3,4,7,8- Hexachlorodibenzo-p-dioxin (HxCDD)[K]	0.002
1,2,3,7,8,9- Hexachlorodibenzo-p-dioxin (HxCDD)	0.002
1,2,3,4,6,7,8- Heptachlorodibenzo-p-dioxin (HpCDD)	0.002
Octachlorodibenzo-p-dioxin (OCDD)	0.001
2,3,7,8-Tetrachlorodibenzofuran (TCDF)	0.008
1,2,3,7,8-Pentachlorodibenzofuran (PeCDF)	0.002
2,3,4,7,8-Pentachlorodibenzofuran (PeCDF)	0.002
1,2,3,6,7,8-Hexachlorodibenzofuran (HxCDF)[L]	0.002
1,2,3,7,8,9- Hexachlorodibenzofuran (HxCDF)	0.002
1,2,3,4,7,8- Hexachlorodibenzofuran (HxCDF)[L]	0.002
2,3,4,6,7,8- Hexachlorodibenzofuran (HxCDF)	0.002
1,2,3,4,6,7,8- Heptachlorodibenzofuran (HpCDF)	0.002
1,2,3,4,7,8,9- Heptachlorodibenzofuran (HpCDF)	0.002
Octachlorodibenzofuran (OCDF)	0.001

DL-PCB: dioxin-like polychlorinated biphenyl
EPA: United States Environmental Protection Agency
GC-ECD: gas chromatography-electron capture detector
GC-HRMS: gas chromatography-high resolution mass spectrometry
MDL: method detection limit
ng/mL: nanogram(s) per milliliter
PCB: polychlorinated biphenyl
PCDD: polychlorinated dibenzo-p-dioxin
PCDF: polychlorinated dibenzofuran



µg: microgram(s)
g: gram(s)
GC-ECD: gas chromatography-electron capture detector
GC-HRMS: gas chromatography-high resolution mass spectrometry
PCB: polychlorinated biphenyl
pg: picogram
TEQ: toxic equivalent
ww: wet weight

Figure A1. Dietary sum TEQ vs sum PCB standard curve used to estimate dietary TEQ concentrations that were not directly measured

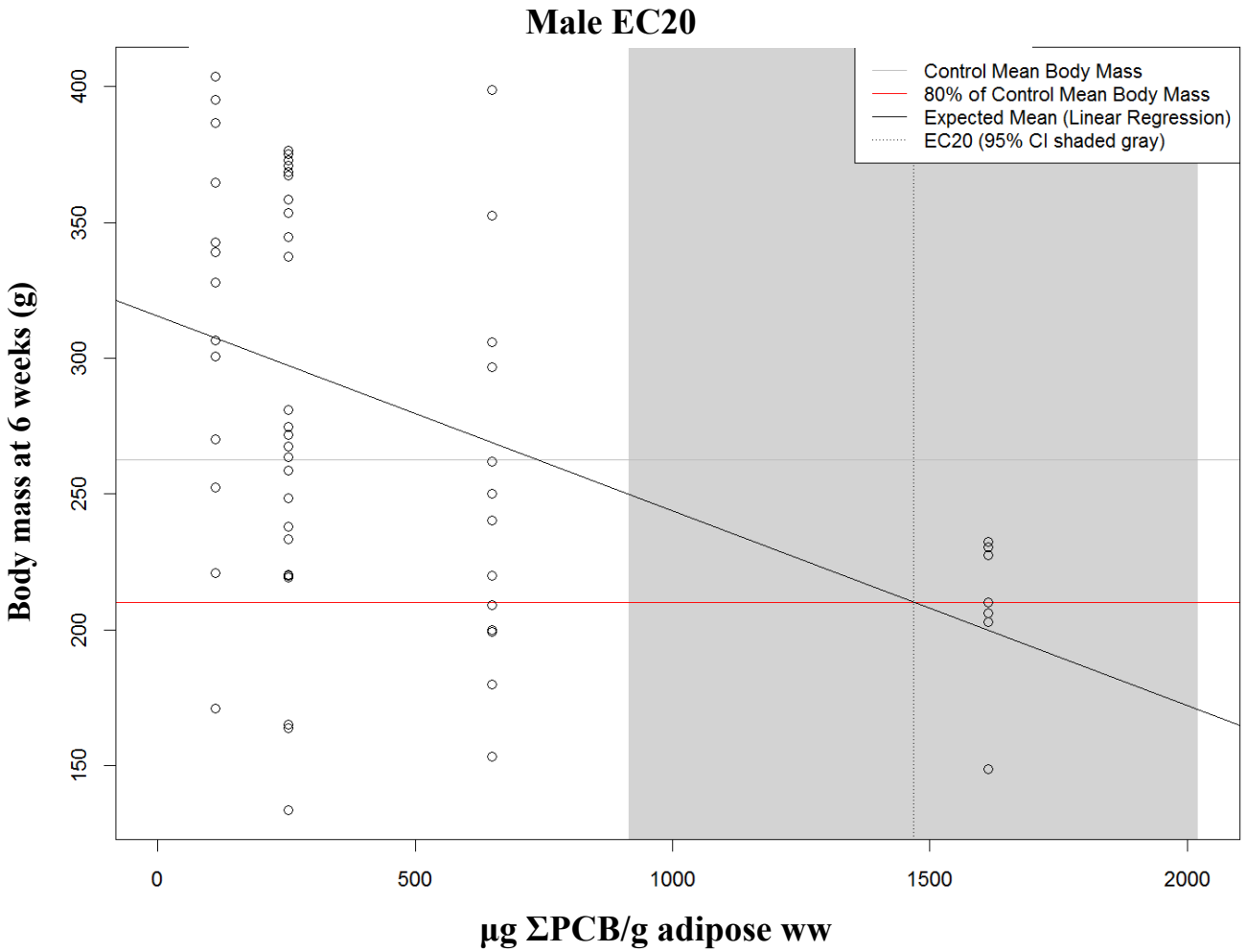


Figure A2. Adipose PCB dose-response curve and an EC20 estimate for male kit mass at 6 weeks of age

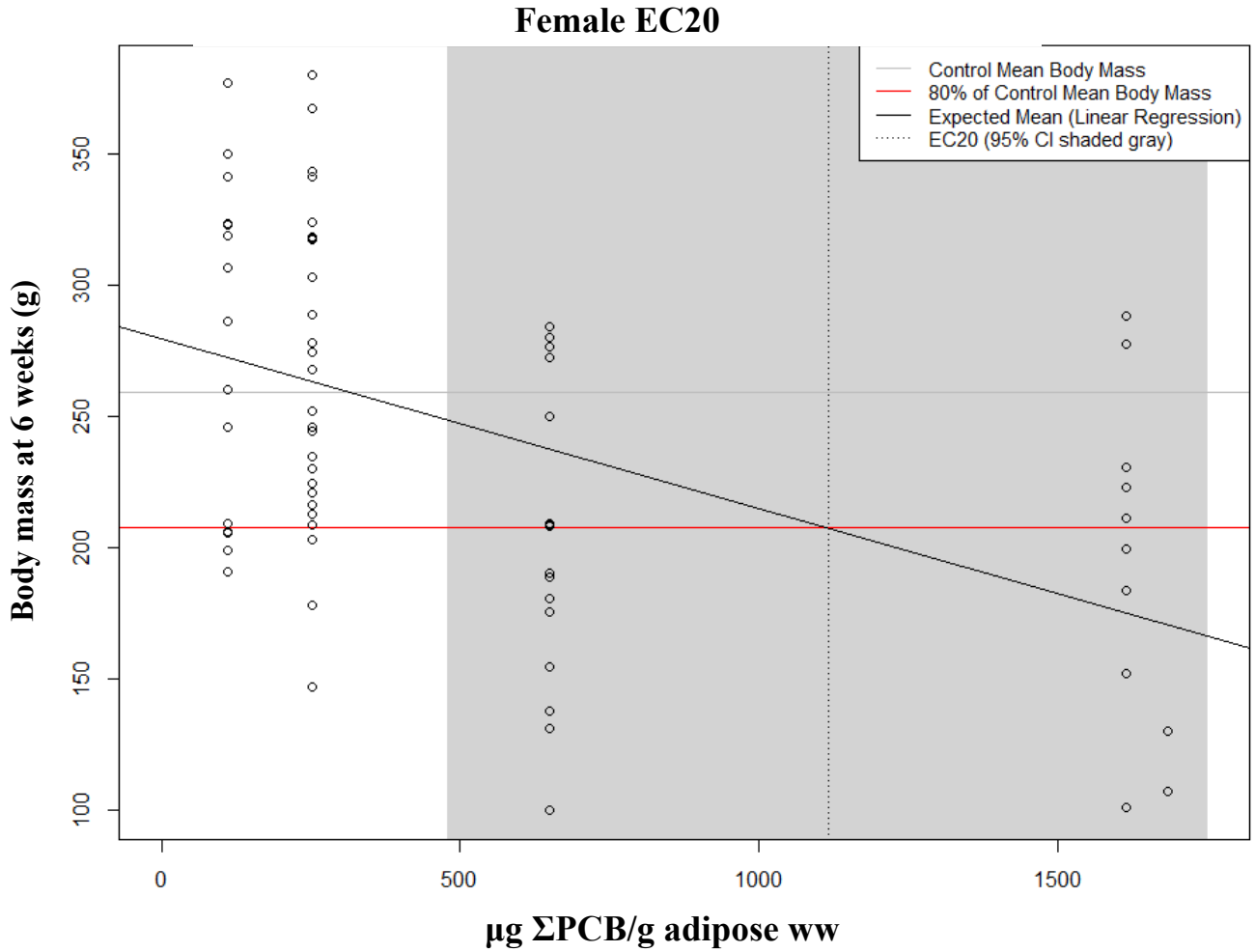


Figure A3. Adipose PCB dose-response curve and an EC20 estimate for female kit mass at 6 weeks of age

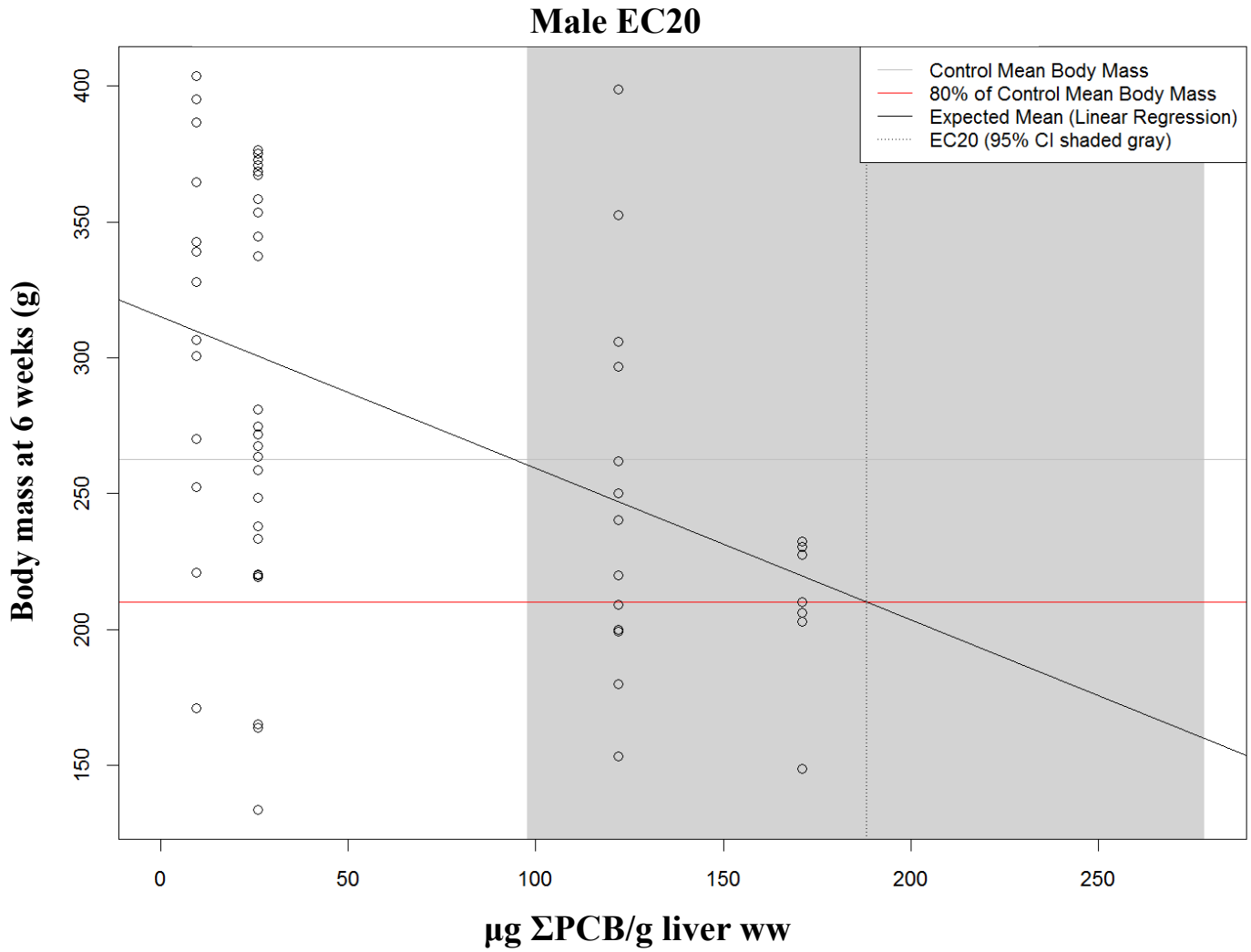


Figure A4. Liver PCB dose-response curve and an EC20 estimate for female kit mass at 6 weeks of age

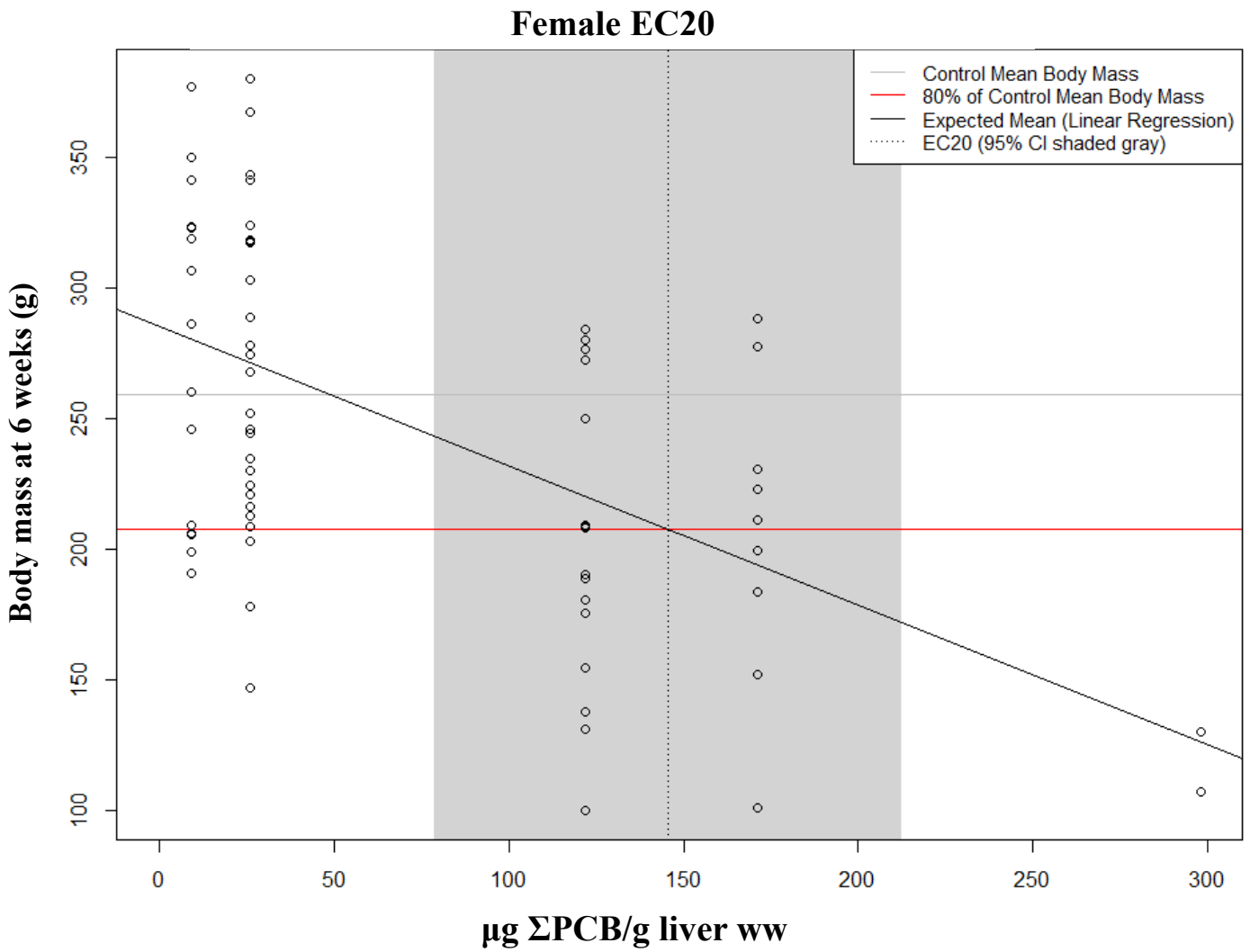


Figure A5. Liver PCB dose-response curve and an EC20 estimate for female kit mass at 6 weeks of age

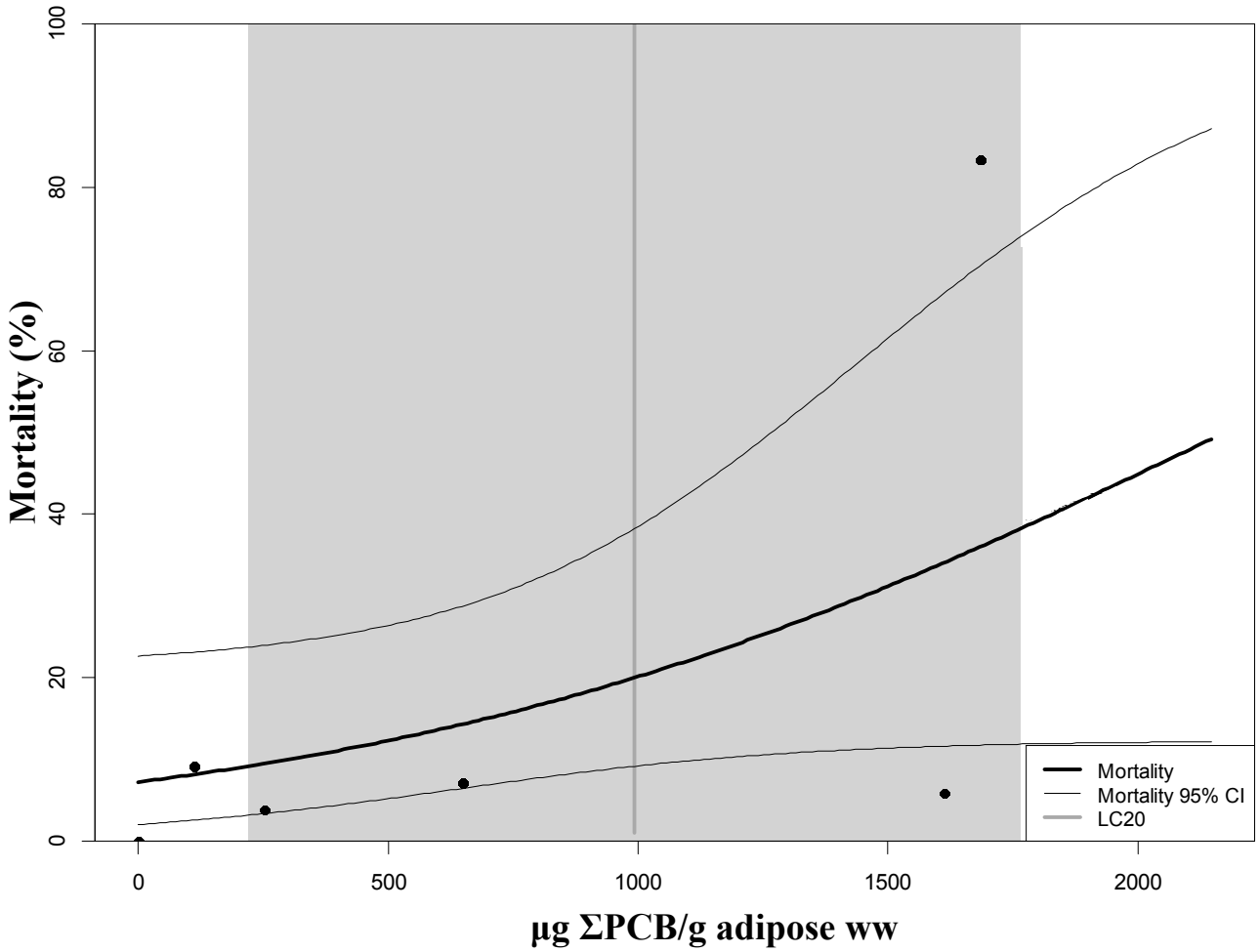


Figure A6. Adipose PCB dose-response curve and a LC20 estimate for kit mortality at 6 weeks of age

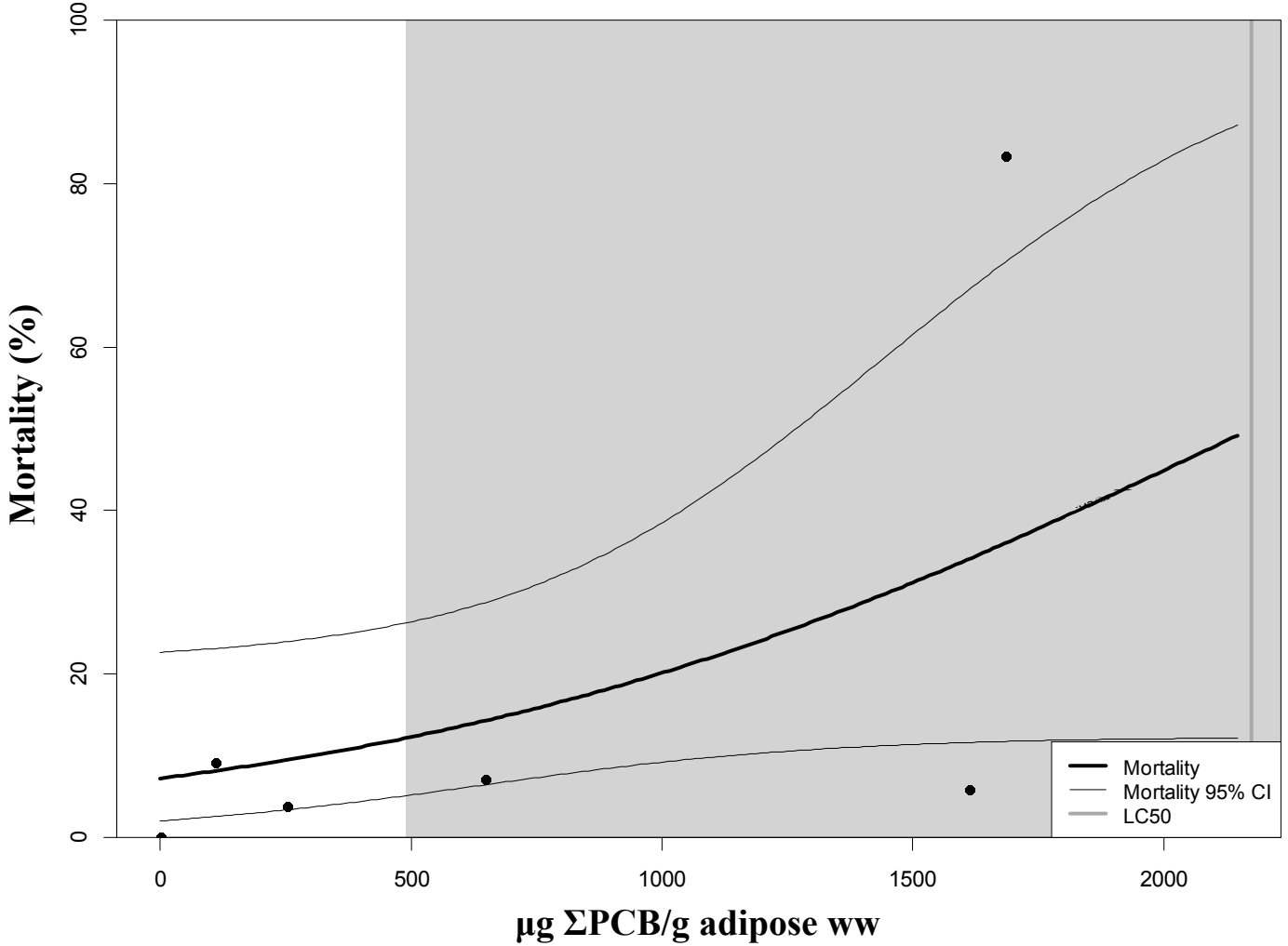


Figure A7. Adipose PCB dose-response curve and a LC50 estimate for kit mortality at 6 weeks of age

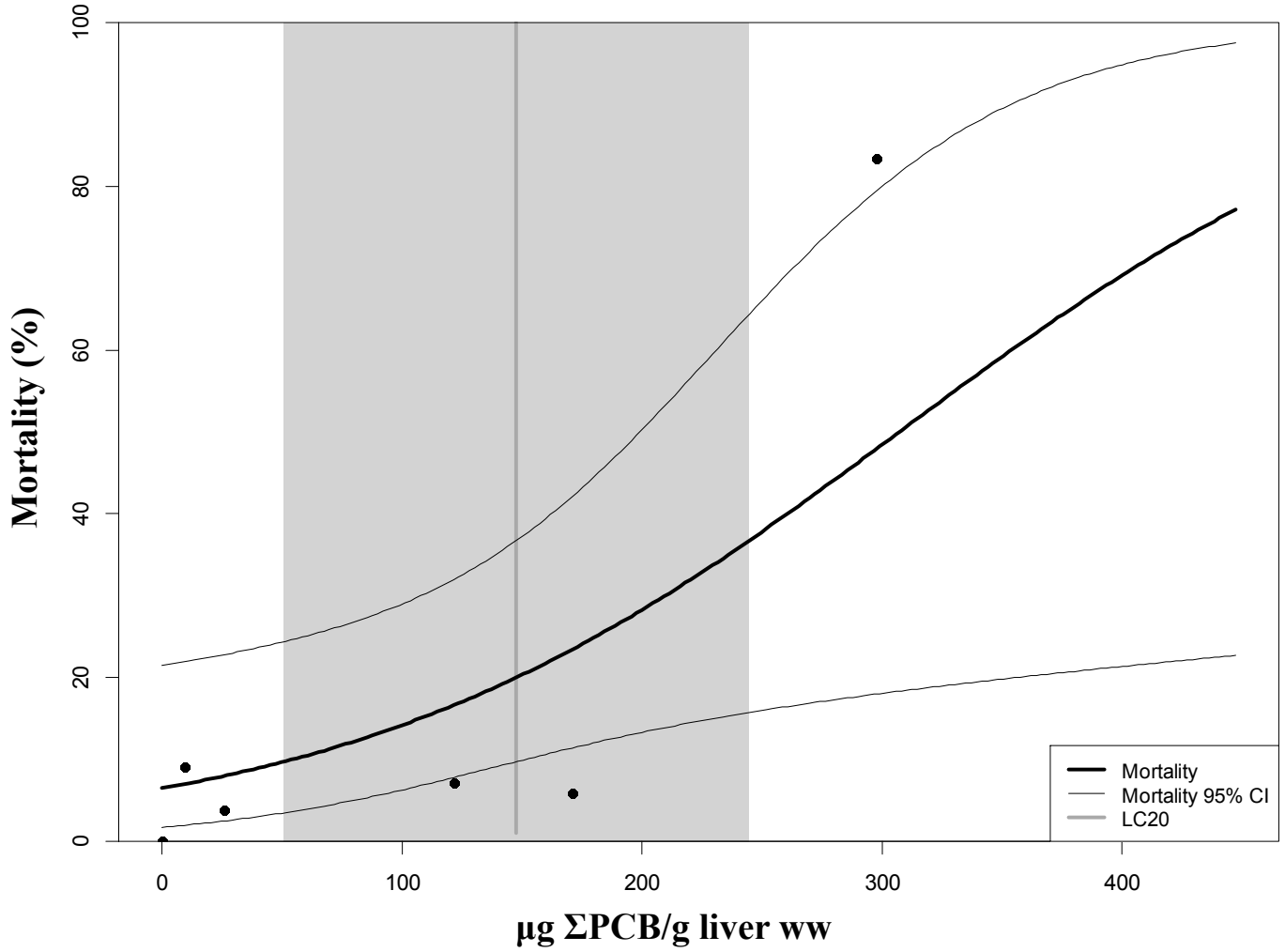


Figure A8. Liver PCB dose-response curve and a LC20 estimate for kit mortality at 6 weeks of age

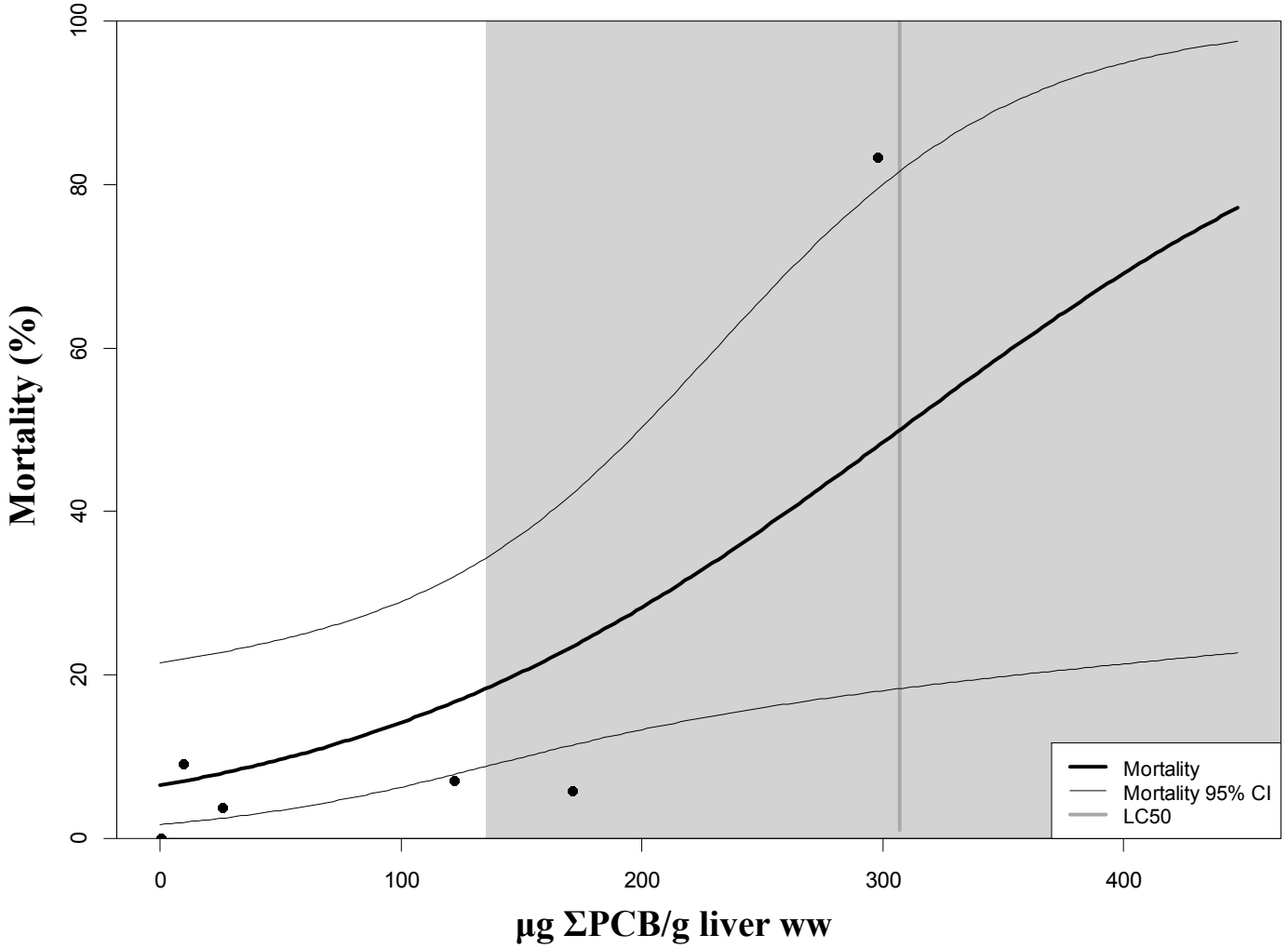


Figure A9. Liver PCB dose-response curve and a LC50 estimate for kit mortality at 6 weeks of age

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LITERATURE CITED

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CHAPTER 3

ENZYME INDUCTION AND HISTOPATHOLOGY ELUCIDATE AHR VS NON-AHR MEDIATED EFFECTS OF AROCLOR 1268 IN AMERICAN MINK (*NEOVISON VISON*)

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ABSTRACT

Potential adverse effects on bottlenose dolphins (*Tursiops truncatus*) from polychlorinated biphenyl (PCB) exposure in the Turtle/Brunswick River Estuary (TBRE, southeastern Georgia, USA) are of concern given the magnitude of concentrations reported in preferred prey and blubber tissues. However, PCBs in TBRE dolphins are primarily derived from Aroclor 1268, and predicting toxic effects of Aroclor 1268 is uncertain due to the mixture's unique composition and associated physiochemical characteristics compared to better studied Aroclor mixtures. These differences suggest toxicity benchmarks identified in studies of other PCB mixtures may not be relevant to TBRE dolphins. American mink (*Neovison vison*) were used as a surrogate model for cetaceans to characterize mechanisms of action associated with exposure to Aroclor 1268. Mink share similarities in phylogeny and life history with cetaceans and are characteristically sensitive to PCBs, making mink an attractive surrogate species for marine mammals in toxicity studies. Adult female mink and an F1 generation were exposed to Aroclor 1268 through diet, and effects on enzyme induction, histopathology, thyroid hormone regulation, and hematology were compared to both a negative control and a positive control of 3,3',4,4',5-pentachlorobiphenyl (PCB 126). Aroclor 1268 dietary exposure concentrations ranged from 1.8 to 29 micrograms (µg) Aroclor 1268 per gram (g) of feed wet weight (ww). Anemia, hypothyroidism, and hepatomegaly were observed in mink exposed to Aroclor 1268. Differences between Aroclor 1268 treatments and the positive control in enzyme induction and the development of squamous epithelial proliferation (SEP) jaw lesions indicate that mechanisms other than

aryl hydrocarbon receptor (AhR)-mediated toxicity are associated with exposure to Aroclor 1268.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are aromatic organohalogen compounds historically used in heat transfer materials, elastomers, sealants, pigments, and waxes. While the inertness and hydrophobicity of PCBs made them ideal for these applications, they also pose an environmental hazard to wildlife at sufficient exposures. As persistent, bioaccumulative environmental contaminants, PCBs can elicit myriad adverse physiological effects. Piscivorous mammals are particularly susceptible due to their elevated potential for exposure as a result of high trophic status and their adipose composition (Tanabe et al. 1994, Safe 1994). Aroclor 1268 is a commercial mixture of PCBs formerly used in fire retardants among other applications, although the mixture was not widely used compared to other Aroclors (Erickson and Kaley 2011, Kannan et al. 1997). Aroclor 1268 was released from a former chlor-alkali facility into the Turtle-Brunswick River Estuary (TBRE, Georgia USA) (Kannan et al.1997). The PCB congener profiles of TBRE bottlenose dolphin (*Tursiops truncatus*) blubber and preferred prey within the estuary are similar to Aroclor 1268 with some differences attributable to background PCBs and environmental weathering (Pulster et al. 2005, Pulster and Maruya 2008). Mean sum PCB (ΣPCB) concentrations in male and female TBRE dolphin blubber of 510 and 116 micrograms (μg) per gram (g) lipid have been reported, respectively (Balmer et al. 2011). These concentrations may exceed adverse effect thresholds derived from the literature (Kannan et al. 2000, Golub et al. 1991,

Schwacke et al. 2002), but the accuracy of these thresholds is uncertain due to the lack of toxicological data specific to Aroclor 1268 that are applicable to marine mammals.

Correlations between Aroclor 1268 exposure and disruption of thyroid hormones (THs) and anemia have been reported in TBRE dolphins (Schwacke et al. 2012). Laboratory studies confirm that various forms of hypothyroidism and anemia are associated with PCB exposure in mammals (Arnold et al. 1993a, Arnold et al. 1993b, Tryphonas et al. 1984, Chu et al. 1994); however, these laboratory studies describe effects from exposure to single PCB congeners or mixtures that are different in congener profile from Aroclor 1268. The few known mammalian toxicity reference values (TRVs) specific to Aroclor 1268 apply to neurotoxic and carcinogenic endpoints and were developed using rodent models (Simon et al. 2007, Warren et al. 2004). Toxicity reference values that apply to a broader scope of endpoints are needed to gain a more comprehensive understanding of mechanisms of toxicity associated with Aroclor 1268 exposure that may be experienced by marine mammals. Similarities in phylogeny, trophic status, and a characteristic sensitivity to PCBs make American mink (*Neovison vison*) a preferred surrogate model for marine mammals when compared to rodents for PCB toxicity studies (Basu et al. 2007). Here, effects of chronic dietary exposure to Aroclor 1268 at concentrations relevant to TBRE dolphins were assessed in a laboratory feeding study that employed mink as a surrogate model. Effects of Aroclor 1268 exposure on TH concentrations, hematological measurements associated with anemia, histopathology, and morphology in mink are described in this study. Effects on growth and reproduction from this study are described in Folland et al. (2015).

Exposure to Aroclor 1268 likely elicits toxic effects through multiple mechanisms of action. Non-ortho substituted PCBs (dioxin-like, DL-PCBs) share structural similarities to polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). These types of molecules produce toxic effects through a shared pathway involving altered gene transcription induced by ligand binding to the aryl hydrocarbon receptor (AhR). In contrast, ortho-substituted (non-dioxin-like) PCBs have little to no binding affinity to the AhR and produce toxic effects through mechanisms that have not yet been fully characterized (Giesy and Kannan 1998 and 2002, Fischer et al. 1998, Safe et al. 1994). Relatively few studies have described effects of non-dioxin-like PCBs in mammalian species, while most have focused on the 12 dioxin-like congeners and commercial PCB mixtures that contain substantial concentrations of these congeners. Aroclor 1268 is atypically dominated by the highly chlorinated hepta through deca PCB congeners. Dioxin-like PCBs are less prevalent in Aroclor 1268 compared to most Aroclor mixtures. Aroclor 1268 typically contains a relatively low proportion of 3,3',4,4',5 pentachlorobiphenyl (PCB 126) (Maruya and Lee 1998, Rushneck et al. 2004), the most potent AhR inducing DL- PCB congener (Van den Berg et al. 2006). Application of the World Health Organization (WHO) 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) equivalent (TEQ) approach for measuring the contribution of PCDDs/PCDFs and DL-PCBs to total toxicity suggests adverse effect threshold concentrations are greater (i.e., less toxic) for Aroclor 1268 than many of the more widely used Aroclors (Rushneck et al. 2004, Burkhard and Lukasewycz 2008). However, the TEQ approach assumes DL-PCBs cause critical effects at lower concentrations than non-dioxin-like PCBs and does not accommodate mechanisms of action that differ from the AhR mediated pathways.

There are surely other mechanisms of toxicity induced by the congeners in Aroclor 1268. Since the mixture typically has low relative abundances of dioxin-like molecules that induce toxic effects at lower threshold concentrations than ortho-substituted PCBs and mask non-AhR mediated toxicity, this study provides a unique opportunity to evaluate AhR vs non-AhR mediated PCB toxicity.

Toxic effects in mink exposed to Aroclor 1268 were compared to a positive control group chronically exposed to PCB 126 to differentiate AhR versus non-AhR mediated effects. Polychlorinated biphenyl 126 was selected as a positive control because it is the most potent inducer of the AhR pathway of all PCBs and its toxicity is the most well defined of all PCB congeners (Giesy and Kannan 1998, 2002). Two biomarkers of AhR activity were measured in Aroclor 1268 and PCB 126 treated mink: development of squamous epithelial proliferation (SEP) jaw lesions and cytochrome P450 enzyme activity. Nests and cords of squamous epithelial cells form in the gingiva of mink exposed to DL-PCBs. Depending upon the degree of exposure, these neoplasia can progressively extend into the alveolar bone, resulting in osteolysis of the skull and eventual tooth loss in both mink and marine mammals (Render et al. 2000a, 2000b; Bergman et al 1992a). In mink, SEP jaw lesions appear histologically at exposures 10 to 40 fold lower than those associated with reproductive effects on a total TEQ basis, making SEP jaw lesions a useful diagnostic tool for identifying AhR pathway induction at low exposures (Beckett et al. 2005, Bursian et al. 2006, Moore et al. 2009). Additionally, dioxin-like molecules induce cytochrome-P450 1A1 (CYP1A1) enzyme activity via the AhR pathway, while little to no CYP1A1 response is elicited by exposure to non-dioxin-like PCBs (Denison and Nagy 2003, Haynes et al. 2009). Here, adverse

effects observed in mink treated with Aroclor 1268 are compared with effects in PCB 126 treated mink, and biomarkers of AhR activity are used to validate the presence of AhR-mediated toxicity by treatment, enabling differentiation of toxicity manifested through different mechanisms.

MATERIALS AND METHODS

Experimental design (see CHAPTER 1 Materials and Methods for additional details)

Concentrations of PCBs in treatment diets administered to mink were designed to simulate realistic and extreme exposure scenarios experienced by TBRE bottlenose dolphins by bracketing and exceeding bottlenose dolphin preferred prey collected from TBRE. Adult female mink and kits (through weaning) were given treatment diets spiked with Aroclor 1268 neat material at concentrations of 1.77, 4.01, 10.6, 17.1, and 28.8 µg sum PCB (ΣPCB)/g feed wet weight (ww). The positive control diet spiked with PCB 126 that was administered to adult female mink and kits contained 90.2 picograms (pg) ΣTEQ/g feed. Aroclor 1268 treatment diets given to juveniles from post-weaning through 27 weeks of age were 1.05, 3.10, 8.10, 16.8, and 28.8 µg ΣPCB/g feed ww (ΣTEQ concentrations given in Tables 1 and 2). The positive control diet administered to juvenile mink was 131.5 pg ΣTEQ/g feed (Tables 1 and 2). The concentrations of PCB 126 in the positive control diets were selected based on similar ΣTEQ concentrations that adversely affected reproduction and growth without inducing total reproductive failure (Zwiernik et al. 2009).

Mink were housed individually in wire cages aligned in two adjacent facing rows in an outdoor, partially-open structure at the Michigan State University (MSU) Experimental Fur Farm. The MSU Institutional Animal Care and Use Committee approved study design and animal husbandry practices (AUF#11/11-230-00). Seventy adult female mink were randomly divided into a negative control, positive control (PCB 126), and 5 Aroclor 1268 treatment groups of 10 individuals each. Treatment diets were mixed based on a standard ranch formulation designed to meet nutritional requirements of mink (Folland et al. 2015). Treatment diets were spiked with one of five increasing amounts of Aroclor 1268 or a single concentration of PCB 126 neat material dissolved in soybean oil. Mink were provided 175 g of feed daily to simulate a continuous, chronic dietary exposure scenario throughout the study. Treatment diet feeding commenced in February 2012. Females were weighed every three weeks except during gestation. Adult females were paired with male mink for breeding during March 2012. Three copulation events were confirmed per female. Pregnant females (dams) gave birth to (whelped) kits after an average 47-day gestation period. Dams and respective litters were housed together during lactation and weighed 24 hours postwhelp and when kits reached 3 and 6 weeks of age. Kits were weaned from dams at 6 weeks of age. Necropsies of all dams and a random subset of kits were performed at weaning in June 2012. A random subset of weaned kits (juveniles) was selected to remain on treatment diets until 27 weeks of age. The remainder of the kits were euthanized, archived as whole body samples, and stored at -25°C. Treatment diets were mixed in January 2012 and June 2012. The first batch was administered to adult females and kits until weaning when postweaned juvenile mink were transitioned to the second feed batch.

Necropsies of juvenile mink were performed in November 2012 at the conclusion of the exposure period.

Dose-dependent poor palatability of Aroclor 1268 treatment diets resulted in decreased feed consumption and diminished nutritional state in some individuals. These individuals could not be sustained on treatment diets until necropsy; hence some sample sizes were reduced. Six of 10 adult females in the 28.8 µg ΣPCB/g feed ww treatment could not be sustained on treatment diets and were euthanized in accordance with institutional animal care and use guidelines, resulting in a treatment sample size of 4. One adult female in the 1.77 µg ΣPCB/g feed ww treatment and 2 juvenile mink (negative control and 8.10 µg ΣPCB/g feed ww treatments) experienced mortality unrelated to treatment and were excluded from analyses. A detailed explanation of these results, their relationship with growth and reproductive endpoints on Aroclor 1268 treated mink, and implications on statistical interpretations is provided in Folland et al. (2015).

Necropsy

Dams and juveniles were anesthetized with ketamine (1 milliliter per kilogram [mL/kg] body weight [wt]) prior to necropsy, and blood was collected via cardiac puncture for plasma PCB analysis and TH and complete blood count (CBC) profiles. Blood was not collected from kits due to insufficient blood volume for PCB and TH analysis. Mink were euthanized by carbon dioxide (CO_2) asphyxiation and necropsies were performed immediately following blood collection. One-gram aliquots of liver were removed within five minutes of expiration and preserved in liquid nitrogen for

subsequent analysis of ethoxyresorufin-O-deethylase (EROD) activity. An American College of Veterinary Pathologists board certified pathologist examined carcasses and organs for gross morphological abnormalities. Liver, kidneys, spleen, thymus, heart, adrenal glands, thyroid glands, brain, maxilla, and mandibles were removed, weighed, and preserved in 10% neutral buffered formalin. Tissues collected for PCB, PCDD, and PCDF analyses included liver and adipose tissue from dams, kits, and juvenile mink, as well as plasma from dams and juveniles. All necropsy instruments were rinsed with solvents to avoid cross-contamination, and all samples for analytical chemistry were stored in certified contaminant-free glass containers.

Organs were weighed to the nearest 0.0001g during necropsy. Comparative analyses were based on the masses of organs relative to the individual's brain weight. Brain mass was chosen as the preferred normalizing metric over body mass because brain mass is strongly correlated with body size and less temporally variable in mink than body mass, which fluctuates greatly by season (Kruska 1996). Relative measurements of brain masses were obtained by normalizing to body length.

Analytical chemistry

PCBs by gas chromatography-electron capture detector (GC-ECD) (Modified from EPA Method 8082): Polychlorinated biphenyl congeners were quantified in diet, liver, adipose, and plasma samples. Samples were homogenized with anhydrous sodium sulfate and spiked with [^{13}C] PCB surrogate standards (Accustandard). Polychlorinated biphenyls were separated from samples with an 18-hour soxhlet extraction using 400 mL of 3:1 dichloromethane:hexane. Extracts were dried, and lipid

content was quantified. Lipids were resuspended in hexane, and the solution was cleaned with sulfuric acid and an acidic/neutral silica gel column. Extracts were concentrated to one mL, and 61 PCB congeners were quantified by GC-ECD (Hewlett Packard 5890 Series II) at the MSU Wildlife Toxicology Laboratory. Quality assurance-quality control samples (QA-QC) were analyzed for every batch at a 20-sample frequency. Quality assurance-quality control samples included laboratory blank, laboratory control sample, matrix spike, matrix spike duplicate, and standard reference material (SRM). Lake Superior fish and whale blubber (National Institute of Standards and Technologies) were used as SRMs to validate PCB analytical chemistry. Congener-specific method detection limits (MDLs) were determined at the beginning of the study (Table A1). A proxy value of one-half the MDL concentration was applied to PCB congeners that were neither detected nor detected below the MDL. Concentrations are expressed on wet-weight (ww) basis unless otherwise noted.

Dioxins, furans, and dioxin-like-PCBs by gas chromatography-high resolution mass spectrometry (GC-HRMS) (Modified from EPA Methods 1613 and 1668):
Polychlorinated dibenzo-p-dioxins (PCDDs), PCDFs, and DL-PCBs were extracted from diet, liver, and adipose samples using a soxhlet extraction method similar to that described above with modifications. Samples were spiked with [^{13}C] PCB and PCDD/PCDF surrogate standards (Wellington). Soxhlets contained 400 mL of dichloromethane, and an additional carbon column cleanup step was performed to fractionate ortho-substituted PCBs in hexane from non-ortho PCBs, PCDDs, and PCDFs using toluene. Extracts were concentrated by nitrogen gas stream in 100

microliters (µL) of tetradecane in 250 µL microvials. Quality assurance-quality control sample types and frequencies were similar to samples extracted for analysis by GC-ECD. Ten PCDF, 7 PCDD, and 4 non-ortho-substituted PCBs (Table A1) were quantified in extracts by GC-HRMS (Thermo Scientific Ultra Trace/ ZB-5MSi Zebron® capillary column [30 m×0.25 mm×0.25 µm], Phenomenex; Torrance, CA, USA) at the Wadsworth Center (Albany, NY, USA). Sum TEQ values were calculated by summing the products of the concentrations of 17 PCDD/PCDF and 4 non-ortho-substituted PCB congeners with their respective mammalian 2005 World Health Organization 2,3,7,8 TCDD Equivalent Factors (WHO_{2005}TEFs) (Van den Berg et al. 2006). Estimated ΣTEQ values based on a linear standard curve between ΣTEQ vs ΣPCB concentrations (R^2>0.999) were applied to dietary samples for which GC-HRMS analysis was unavailable (Figure A1).

Thyroid hormone analyses

Blood was collected from dams and juvenile mink in Vacutainers® that contained no anticoagulant. Blood samples were kept at room temperature for 30 minutes to allow clotting before centrifugation (20 min × 1,000 *g*) at 4°C. The supernatant was pipetted into 5 mL vials and stored at -25°C for no more than one week before delivery to the MSU Diagnostic Center for Population and Animal Health (DCPAH) Endocrine Section for TH profile analysis of 3,5,3',5' tetraiodothyroxine (T4) and 3,5,3' triiodothyronine (T3). Total T4 (TT4), total T3 (TT3), free T4 (FT4), and free T3 (FT3) were measured in all samples with sufficient volume. TT4 was measured using the Gamma Coat M Total T4 kit (DiaSorin), TT3 was measured with TT3 antibody (MP Biomedical) and ^{125}I T3 kits

(New England Nuclear), and FT3 was measured using the Clinical Assays GammaCoat Free T3 ^{125}I RIA kit (DiaSorin). Samples were sent to Antech Diagnostics for FT4 quantification by equilibrium dialysis. Detailed procedures for these methods are available directly from MSU DCPAH. All 4 methods were validated for precision and accuracy using 10 replicates from 3 pooled samples. Inter/intra coefficient of variations of all 4 methods and R^2 values fell within acceptable ranges (CV: 7%-16%, R^2: 0.93-0.98).

Hematology

Whole blood samples were collected from dams and juvenile mink in Vacutainers® (Becton, Dickinson, and Co.) containing ethylenediaminetetraacetic acid preservative to minimize clotting. Samples were transported on ice to the DCPAH Clinical Pathology Section (East Lansing, MI, USA) and analyzed the same day as collected. Whole blood samples from dams and juvenile mink that contained sufficient volume (≥0.25 mL) and passed quality control criteria for clotting and hemolysis were included in CBC profile analyses. Red blood cell (erythrocyte) count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume of erythrocytes (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), hemoglobin distribution width (HDW), platelet count (PLT), mean platelet volume (MPV), white blood cell count (WBC) and total blood protein (TBP) were measured using an ADVIA 120 Hematology System (Siemens, Munich Germany). Measurements were selected based on relevance with respect to

anemia observed in TBRE dolphins and laboratory animals in PCB exposure studies (Schwacke et al. 2012, Arnold et al. 1993a, Tryphonas et al. 1984, Chu et al. 1994).

Histopathology

Histopathological examination of organs and jaws were performed without reference to treatment and in no particular order between treatments, age or sex. Liver, spleen, kidneys, adrenal glands, and thyroid glands were examined from dams and kits. A lack of treatment-related abnormalities in these age classes determined additional histopathology analyses of juvenile mink were not needed. Organs were inspected for gross abnormalities before cross-sections were cut and stained with hematoxylin and eosin on slides for microscopy. Maxillae and left and right mandibles from juvenile mink were placed in decalcifying solution for 72 hours to allow sectioning. Adult female and kit mink jaws were not examined for SEP jaw lesions because the lesions are progressive; juvenile mink experienced the longest exposure duration and provided the best opportunity to observe this effect. Maxillae and mandibles were sliced below the gum line to expose lateral cross-sections of teeth, gingiva, and alveolar bone. Sectioned maxillae and mandibles were stained with hematoxylin and eosin on microscopy slides and examined for SEP jaw lesions. Squamous epithelial proliferation jaw lesions were characterized using a three-tiered categorical scale for severity. One to two small SEP lesions limited to one portion of the dental arcade were described as mild. Two to six larger lesions that were not restricted to one area of the dental arcade were characterized as moderate. Jaws with greater than six SEP lesions widespread

throughout the dental arcade were termed severe. Severe lesions are large and coalescing and are often associated with significant bone loss.

EROD assay

Hepatic microsome preparation: One-gram samples were collected from the right lobes of livers within five minutes of euthanization, immediately prior to necropsy. Samples were placed in cryogenic vials (Corning) and stored in liquid nitrogen. Hepatic microsomes were prepared from 6 individuals per treatment and age class (where sample size permitted) using a method modified from Moore et al. (2009). Samples were homogenized in Tris buffer and transferred to centrifuge tubes. Centrifuge tubes were brought to 5 mL with microsomal stabilizing buffer, and the cellular fraction was separated by centrifugation. Hepatic microsomes were then separated from the cytosolic supernatant by ultracentifugation. Microsomal pellets were resuspended in microsomal stabilizing buffer, divided into three aliquots, and stored at -80°C.

CYP1A1 quantification: CYP1A1 enzyme activity was measured in hepatic microsomes prepared from study mink by quantifying EROD activity following methods modified from Kennedy and Jones (1994) and Moore et al. (2009). One µL aliquots of hepatic microsomes were transferred to 96-well microtiter plates (Corning) containing 80 µL HEPES buffer (0.05 molar (M), pH 7.8) and 30 µL 7-ethoxyresorufin (876 micromolar [µM] in methanol) per well. Plates were incubated for 10 min at 37 °C before the fluorescence reaction between hepatic microsomal CYP1A1 enzymes and 7-ethoxyresorufin was initiated with NADPH. Fluorescence was measured in a Fluoroskan Accent FL microplate fluorometer (Thermo Scientific Waltham, MA, USA).

The reaction was terminated with 60 µL of fluorescamine (0.4 millimolar [mM] in acetonitrile) and absorbance was measured for 10 minutes to quantify protein content. EROD activity and protein concentration were estimated using standard curves generated from fluorescence and absorbance of 0-24 µL resorufin (7.5 µM in methanol) and 0-32 µL bovine serum albumin (2mg/mL HEPES buffer). Estimates of EROD activity and protein content were derived from standard curves that passed data quality criteria ($R^2 \geq 0.93$).

Statistical analysis

Endpoints were considered adversely affected if Aroclor 1268 treatment means were statistically different than the negative control in a dose-dependent manner or if there was a significant linear relationship between diet/tissue PCB concentration and the measurement endpoint. Statistical methods for assessing endpoints were chosen based on the classification of each data type as continuous (contaminant concentrations, EROD activity, organ masses) or binary (jaw and organ histopathology). All analyses were performed using Statistical Analysis software (Version 9.4, SAS Institute Inc.). General linear model (GLM) regression and analysis of variance (ANOVA) coupled with Dunnett's Test for least squares means treatment differences from the negative control were applied to all continuous variable endpoints to identify effects of PCB exposure and treatment differences from the negative control (PROC GLM). Model assumptions were validated prior to application. Linearity and normality of response variable distributions were examined graphically and tested by Shapiro-Wilk ($\alpha=0.05$). The homogeneity of variances assumption was tested visually and by Levene

(α=0.05). The Durbin-Watson statistic was used to identify deviations from independence between data points. Box Cox power transformations (PROC TRANSREG) were used to transform response variables when violations of model assumptions were identified. Analyses proceeded for response variables when model assumptions were satisfied or the data could be reasonably transformed to meet assumptions. Treatment differences for binary variables were identified using Fisher's Exact Test (PROC FREQ). Hematology data did not satisfy linear model assumptions of normality regardless of transformations. Non-parametric Kruskal-Wallis tests (PROC NPAR1WAY) were employed to test differences in hematology measurements between treatments and the negative control group.

Regressions and treatment differences with dietary ΣPCB concentration, as well as regressions with adipose ΣPCB concentration were performed for all endpoints because preferred prey and adipose tissue are more easily attainable from marine mammals than liver; hence, providing more usability. Furthermore, adipose tissue seemed to be less affected by differential accumulation of congeners than liver (see Folland et al. 2015) and may provide a better reflection of exposure when mink dietary and adipose concentrations were at equilibrium. Regressions with hepatic ΣPCB concentration were performed solely for EROD endpoints and relative liver mass because the concentrations of PCBs are particularly relevant for these responses, and the high degree of correlation between adipose, liver, and plasma ΣPCB concentrations make these dependent variables largely redundant. Plasma ΣPCB concentrations were used for TH analyses because these variables interact directly through competitive inhibition of TH transport proteins and deionase enzymes (Brouwer et al. 1989).

Although the sites of measurement were identical (blood), plasma ΣPCB comparisons were not considered in the analysis of hematological parameters because relationships between these measures are not necessarily directly related, but rather involve physiological responses throughout the body that are more appropriately considered in terms of dietary and adipose ΣPCB concentrations.

To account for the confounding factor of nutritional status in determining treatment-related effects, body condition index (BCI) was used as a surrogate metric for nutritional state to provide a post hoc metric for statistical validation of affected endpoints. Body condition index was calculated for dams, kits, and juvenile mink using the value of the individual's residual distance from the ordinary least-squares line of best fit for body mass (g) versus body length (from head to base of the tail, cm) (Schulte-Hostedde et al. 2001, 2005, Folland et al. 2015). Negative residual values (below the line of best fit) represented body masses less than the central tendency for animals of equal body length and were assumed to indicate compromised nutritional state. Body masses 24 hours postwhelp were used to calculate BCI for dams because this time point provided a representation of nutritional state entering lactation. Kits begin to augment an obligate lactate-based diet with administered feed as early as two weeks of age, so the body conditions of dams at whelp are the most relevant with respect to lactation output and ability of a dam to support a litter. Body condition indices could not be appropriately applied to kits because the 6-weeks of age time point coincides with a rapid growth phase when body lengths of individuals may not be comparable, so the BCI value of each kit's respective dam was substituted. Mink body length is typically constant by 27-weeks of age, so BCI values for juvenile mink were

calculated using body mass at necropsy because this is when most endpoints were measurements.

Traditional mixed model statistical approaches for partitioning effects between covariates could not be appropriately applied because a non-independent relationship existed between treatment and BCI, and such models are not equipped to cope with multicollinearity between independent variables. As an alternative approach, response variables that were significantly related to PCB concentration were subsequently tested against BCI using the same statistical procedures. Response variables that were subsequently identified as significantly related to BCI were potentially influenced by nutritional state. In these cases the sensitivity of response variables to BCI was examined by excluding individuals with negative BCI values or treatments with mean BCI values less than the negative control. If the originally significant p-value became less than 0.05, the relationship could not be differentiated between nutrition or toxicity driven, and the result is reported with the disclaimer "ø." A statistically significant relationship between the response variable and treatment when nutritionally compromised individuals were removed is strong evidence that the observation was driven by a toxic effect, and reported with the identifier, ◊. P-values of response variables significantly related to treatment but not BCI are not accompanied by a symbol.

RESULTS

Diet, adipose, liver, and plasma concentrations

Dietary ΣPCB and ΣTEQ concentrations are reported in Tables 1 and 2. Polychlorinated biphenyls 206, 201, and 203 were the most abundant in Aroclor 1268

treatment diets (~33, 14, and 13% of dietary ΣPCBs, respectively). The relative abundances of PCBs 194, 201, 206, and 209 were greater in tissues of all age classes compared to diet. Polychlorinated biphenyl congeners 206 and 201 averaged 57 to 67% of the ΣPCB concentrations in tissues (Figure 1). Polychlorinated dibenzo-p-dioxins and non-ortho-substituted PCBs contributed most to total dietary ΣTEQs (64 and 36%, respectively), while the contribution of PCDFs was negligible (<1%). Eighty-two percent of PCDD derived dietary ΣTEQs were attributed to 1,2,3,7,8 PeCDD, and 96% of non-ortho PCB ΣTEQ were associated with PCB 126 (Table 8).

Table 8. Sum TEQ concentrations measured in dietary treatments[a]

Congener	TEF[b]	Control	1.05 µg ΣPCB/g feed	1.77 µg ΣPCB/g feed	10.6 µg ΣPCB/g feed	28.8 µg ΣPCB/g feed	Adult and kit positive control	Juvenile positivie control
PCDDs								
2378 TCDD	1	ND	ND	ND	ND	ND	ND	ND
12378 PeCDD	1	0.176	1.99	4.75	18.9	52.1	0.232	0.132
123478+123678 HxCDD	0.1	0.00636	0.371	1.20	4.04	10.7	0.0110	0.00573
123789 HxCDD	0.1	ND	0.00248	0.000981	ND	0.0000875	ND	ND
1234678 HpCDD	0.01	0.00139	0.00157	0.00164	0.00126	0.00134	0.00128	0.00128
OCDD[b]	0.0003	0.000373	0.000134	0.000183	0.000133	0.000132	0.00163	0.000188
ΣPCDD TEQs		**0.184**	**2.37**	**5.95**	**22.9**	**62.8**	**0.246**	**0.139**
% contribution of 12378 PeCDD to PCDD ΣTEQs		96	84	80	82	83	94	95
Mean (SD)[c]				**82 (2)**				
% contribution of PCDDs to ΣTEQs		35	38	77	65	74	<1	<1
Mean (SD)[c]				**64 (18)**				
PCDFs								
2378 TCDF	0.1	0.00577	0.00297	0.00614	0.0113	0.0128	0.00526	0.00520
12378 PeDCF	0.03	ND	ND	ND	ND	ND	ND	ND
23478 PeCDF	0.3	ND	ND	ND	ND	0.0215	ND	ND
123478+123678 HxCDF	0.1	0.0171	0.0208	0.0234	0.0461	0.0595	0.0199	0.0161
234678 HxCDF	0.1	0.0154	0.0185	0.0195	0.0262	0.0378	0.0168	0.0163
123789 HxCDF	0.1	0.000719	0.00240	0.00245	0.0110	0.0177	0.000701	0.000681
1234678 HpCDF	0.01	0.000719	0.00240	0.00245	0.0110	0.0177	0.000701	0.000681
1234789 HpCDF	0.01	0.000458	0.000730	0.000586	0.000782	0.00249	0.000673	0.000500
OCDF[b]	0.0003	ND	ND	ND	0.000364	0.000904	ND	ND
ΣPCDF TEQs		**0.0402**	**0.0478**	**0.0545**	**0.1069**	**0.1705**	**0.0440**	**0.0395**
% contribution of PCDFs ΣTEQs		7.6	0.8	0.71	0.30	0.20	<1	<1
Mean (SD)[c]				**0.50 (0.3)**				
ΣPCDD/PCDF TEQs		0.224	2.415	6.003	23.007	62.996	0.290	0.178
% contribution of PCDDs/PCDFs to ΣTEQs		42	39	78	66	74	<1	<1
Mean (SD)[c]				**64 (18)**				
Non-ortho PCBs								
PCB 81	0.0001	ND	0.0272	0.02953	0.3662	0.6994	0.00651	0.00785
PCB 77	0.0003	0.000517	0.0371	0.0343	0.352	0.634	0.00223	0.00349
PCB 126	0.1	0.299	3.75	1.61	11.4	20.7	90	131
PCB 169	0.03	0.00560	ND	0.00558	0.00502	ND	0.191	0.146
ΣNon-ortho PCB TEQs		**0.305**	**3.81**	**1.68**	**12.1**	**22.1**	**89.9**	**131**
% contribution of PCB 126 to non-ortho PCB TEQs		98	98	96	94	94	100	100
Mean (SD)[c]				**96 (2)**				
% contribution of non-ortho PCBs to ΣTEQs		58	61	22	34	26	100	100
Mean (SD)[c]				**36 (18)**				
ΣTEQs		**0.529**	**6.23**	**7.69**	**35.1**	**85.1**	**90.2**	**132**

[a]Units are pg ΣTEQ/g ww, n=1

[b]WHO_{2005} 2,3,7,8 TCDD TEFs from Van den Berg et al. 2005

[b]OCDD, OCDF recoveries<50%

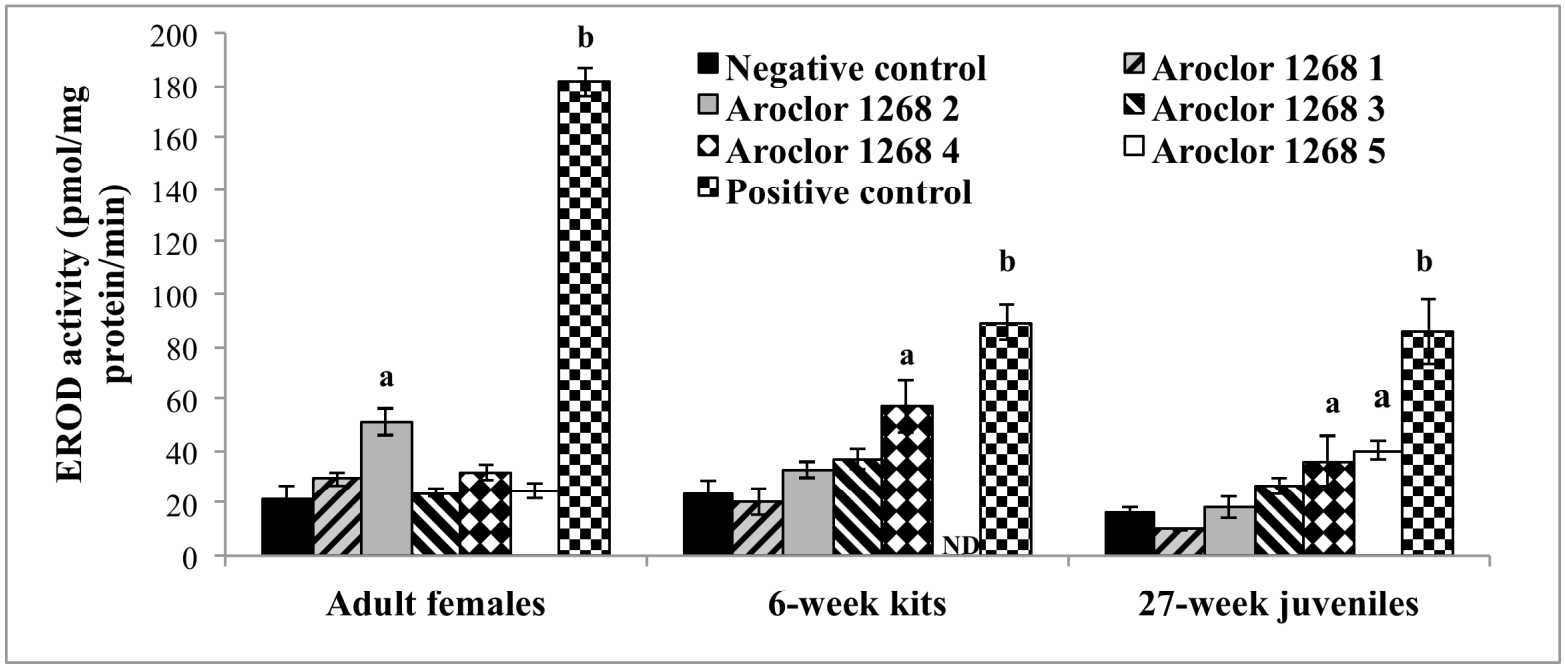
[c]Means are for Aroclor 1268 treatments only

TCDD=tetrachlorodibenzo-p-dioxin; PeCDF=pentochlorodibenzo-p-dioxin; HxCDD=hexachlorodibenzo-p-dioxin; HpCDD=heptachlorodibenzo-pdioxin; OCDD=octachlorodibenzo-p-dioxin; TCDF=tetrachlorodibenzofuran; PeCDF=pentachlorodibenzofuran; HxCDF=hexachlorodibenzofuran; HpCDF=heptachlorodibenzofuran; OCDF=octachlorodibenzofuran. Nomenclature for the PCB congeners follows that of the International Union of Pure and Applied Chemistry: PCB 77=3,3',4,4'-tetrachlorobiphenyl; PCB 81=3,4,4',5-tetrachlorobiphenyl; PCB 126=3,3',4,4',5-pentachlorobiphenyl; PCB 169=3,3',4,4',5,5'-hexachlorobiphenyl

Lipid normalized diet-to-adipose and diet-to-liver biomagnification factors (BMFs) were greater in adult females than juvenile mink. In adult females, BMFs were greatest for intermediate treatments, and were not correlated with dietary ΣPCB concentration ($p>0.05$). Biomagnification factors for juvenile mink were negatively correlated with ΣPCB concentration ($p<0.05$). The lipid-normalized ΣPCB concentration in liver was greater than in adipose for both adult female and juvenile mink (Figure A2).

EROD

Cytochrome p4501A1 (measured by EROD induction) was highly expressed in positive control mink, as expected. EROD activity in the positive control was significantly greater than the negative control and all Aroclor 1268 treatments for all respective age classes ($p<0.001$), with the exception of juvenile mink in the 28.8 µg ΣPCB/g feed treatment ($p>0.05$, Figure 10).



Letters indicate significant differences from the negative control

Groups with different letters have statistically different means

ND: No data

Figure 10. Treatment mean EROD activity in of adult female, 6-week kit, and 27-week juvenile mink

Hepatic EROD activity in adult female mink was not correlated with dietary, adipose, or liver ΣPCB concentrations, while EROD activity correlated significantly with dietary, adipose, and hepatic ΣPCB concentrations in kit and juvenile mink ($p<0.01$).

Histopathology

Percent frequencies (n) of SEP jaw lesions observed in juvenile mink in the negative control and Aroclor 1268 treatments were virtually zero, while jaw lesions were induced in 100% of positive control juvenile mink (Table 9).

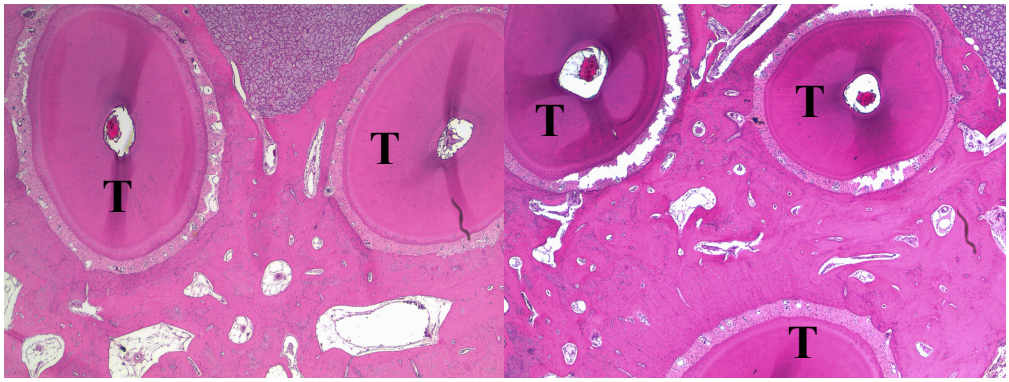
Table 9. Prevalence of SEP jaw lesions in juvenile mink after 27 weeks of dietary PCB exposure

Treatment[a]	n	Individuals with ≥1 lesion	Average severity score	Treatment prevalence (%)
Negative control	9	0	0	0
1.05 µg/g	10	0	0	0
3.01 µg/g	10	1	0.10	10
8.01 µg/g	9	1	0.10	11
16.8 µg/g	10	0	0	0
28.8 µg/g	2	0	0	0
All Aroclor 1268 treatments	41	2	0.024	4.9
Positive control	9	9	2.2	100

[a]µg/g=µg ΣPCB/g feed ww

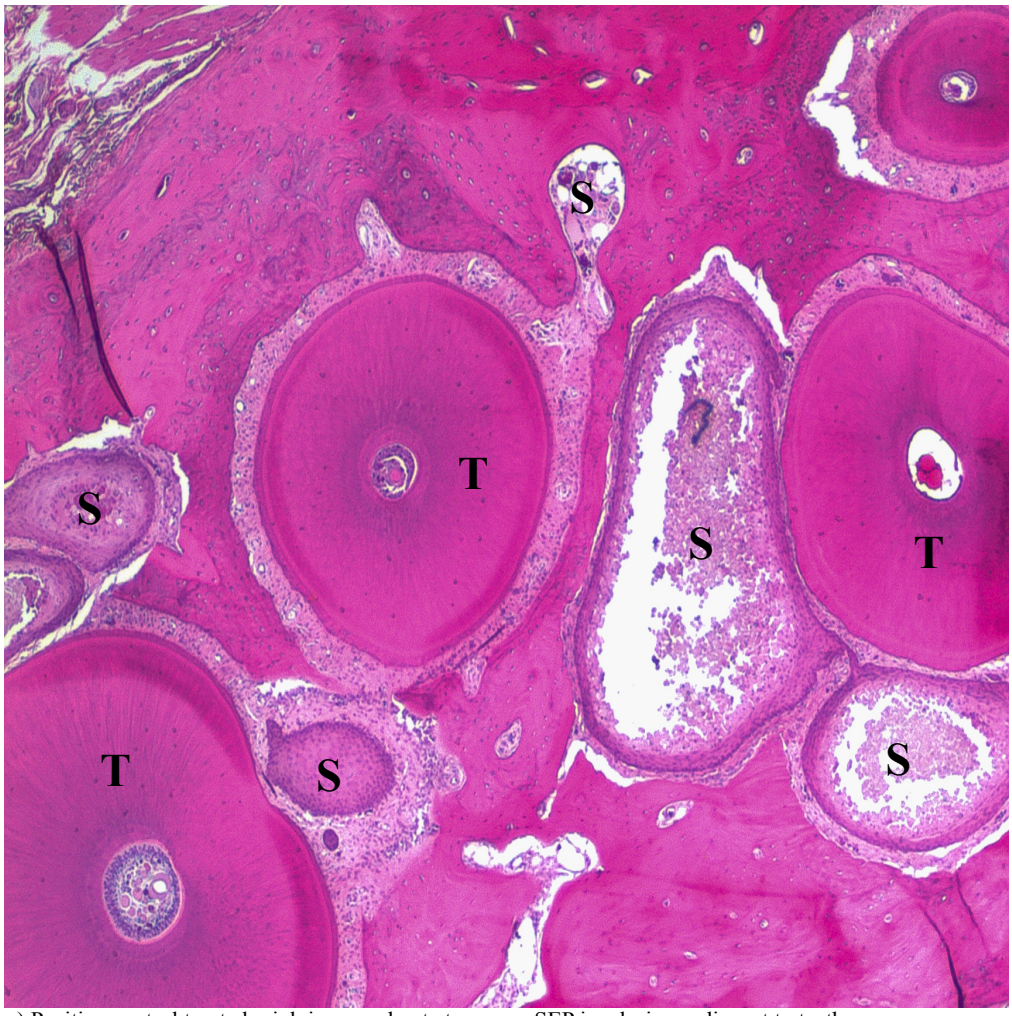
Scoring: 1=mild 2=modeate 3=severe

Jaws of all negative control mink were free of SEP lesions. Jaws of but two Aroclor 1268 treated mink were free of SEP lesions. Lesions in these individuals were early in development, restricted to one region of the right mandible, and characterized as mild. As expected, all mink in the positive control group exhibited moderate to severe jaw lesions that were distributed throughout the dental arcade and had progressed to the stage of some bone loss (Figure 11).



a. Control mink jaw (lesion-free)

b. 28.8 µg ΣPCB/g treatment mink jaw (lesion-free)



c) Positive control treated mink jaw, moderate to severe SEP jaw lesions adjacent to teeth

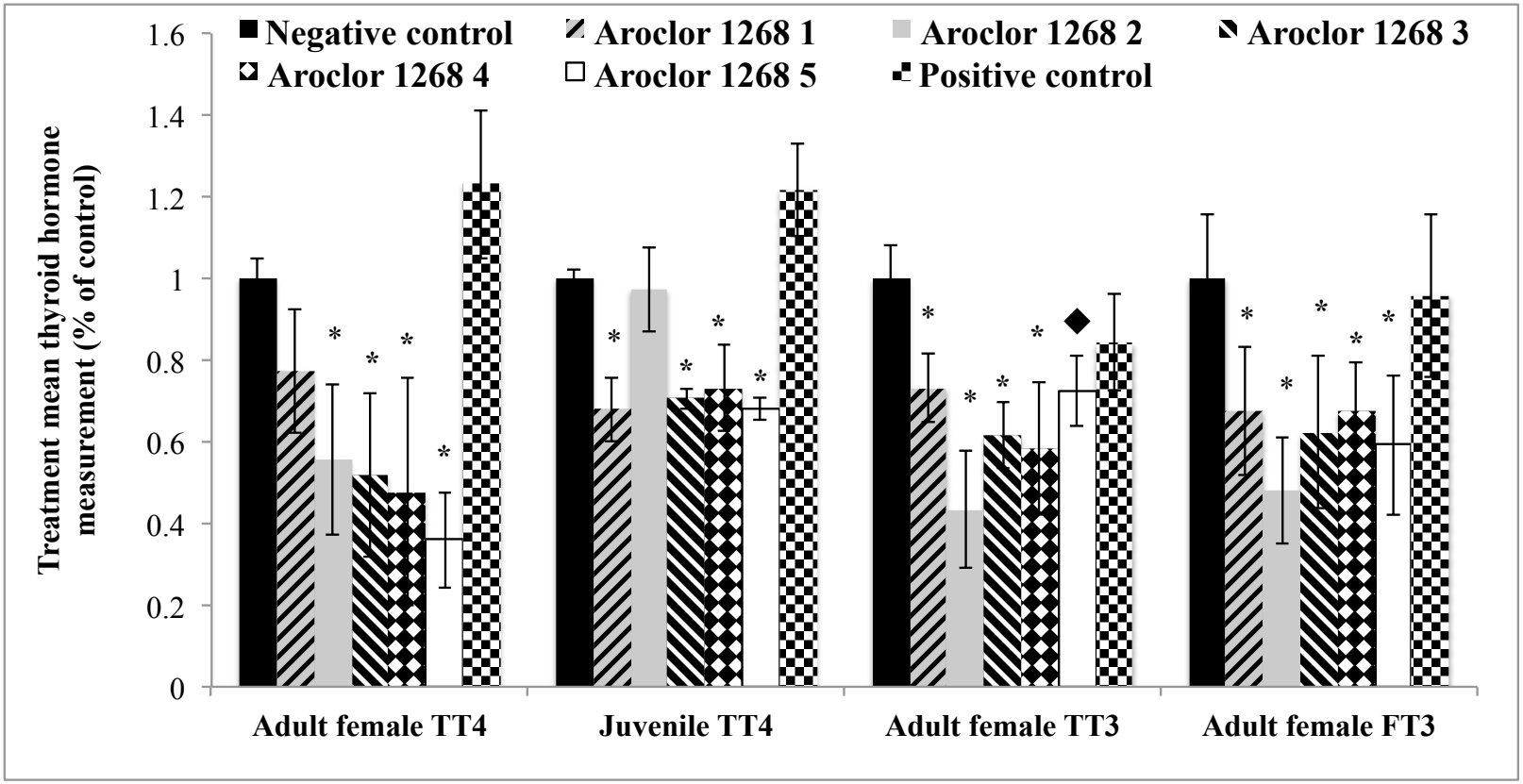
Figure 11. Histology slide images of control (a), Aroclor 1268 (b), and positive control mink (c) mandibles under 2x magnification. (T=teeth, S=SEP lesion)

Histopathological abnormalities were largely absent in organs, and the few notable instances were distributed uniformly across treatments including the negative control. Livers of all adult female and kit mink, including both negative and positive controls, displayed mild to moderate vacuolation of the hepatocellular cytoplasm with no differences across treatments. All spleens of adult female and kit mink were unremarkable with varying amounts of extramedullary hematopoietic tissue. All but two adult female mink displayed trace mineralization of renal tubules, and one adult in the 17.1µg ΣPCB/g feed group displayed unilateral pyelitis and interstitial nephritis. In contrast, kidneys of all but three kit mink were free of mineralization. All adult female and kit mink, including both negative and positive controls, displayed moderate to severe vacuolation of adrenal cortical cells. Adrenal glands of some individuals had scattered aggregates of minerals throughout the adrenal cortex, however frequency was low and not dose-dependent. Thyroid glands of all adult female and kit mink were unremarkable.

Serum thyroid hormone concentrations

Significant correlations between serum TH concentrations and PCB exposure metrics, as well as treatment differences from the negative control indicated that dietary Aroclor 1268 exposure induced hypothyroidism in mink, but exposure to the positive control did not. Plasma ΣPCB concentration seemed to be the best predictor of TH concentrations compared to the other PCB exposure metrics. Plasma ΣPCB concentration tended to yield the lowest p-values and highest R^2 values when correlated with THs with followed by liver, adipose, and dietary ΣPCB concentrations.

Total T4, TT3, and FT3 were lower in adult female and juvenile mink exposed to Aroclor 1268 at various dietary concentrations, while FT4 was unaffected. Total T4 in adult female mink correlated negatively with dietary, adipose, liver, and plasma ΣPCB concentrations, and treatment means decreased dose-dependently from the negative control ($p<0.01$). In juvenile mink, TT4 correlated negatively with liver ΣPCB concentration only, and TT4 treatment means were dose-dependently lower than the negative control ($p<0.05$). Total T3 and FT3 decreased with dietary, adipose, and plasma ΣPCB concentration in adult female mink ($p<0.01$), and Aroclor 1268 treatment means were significantly less than the negative control ($p<0.05$), with the exception of TT3 in mink exposed to 28.8 ($p<0.07$). Both TT3 and FT3 decreased significantly with liver ΣPCB concentration in juvenile mink ($p<0.05$), although treatment differences did not exist ($p>0.05$) (Figures 12, 13, 14, and 15).

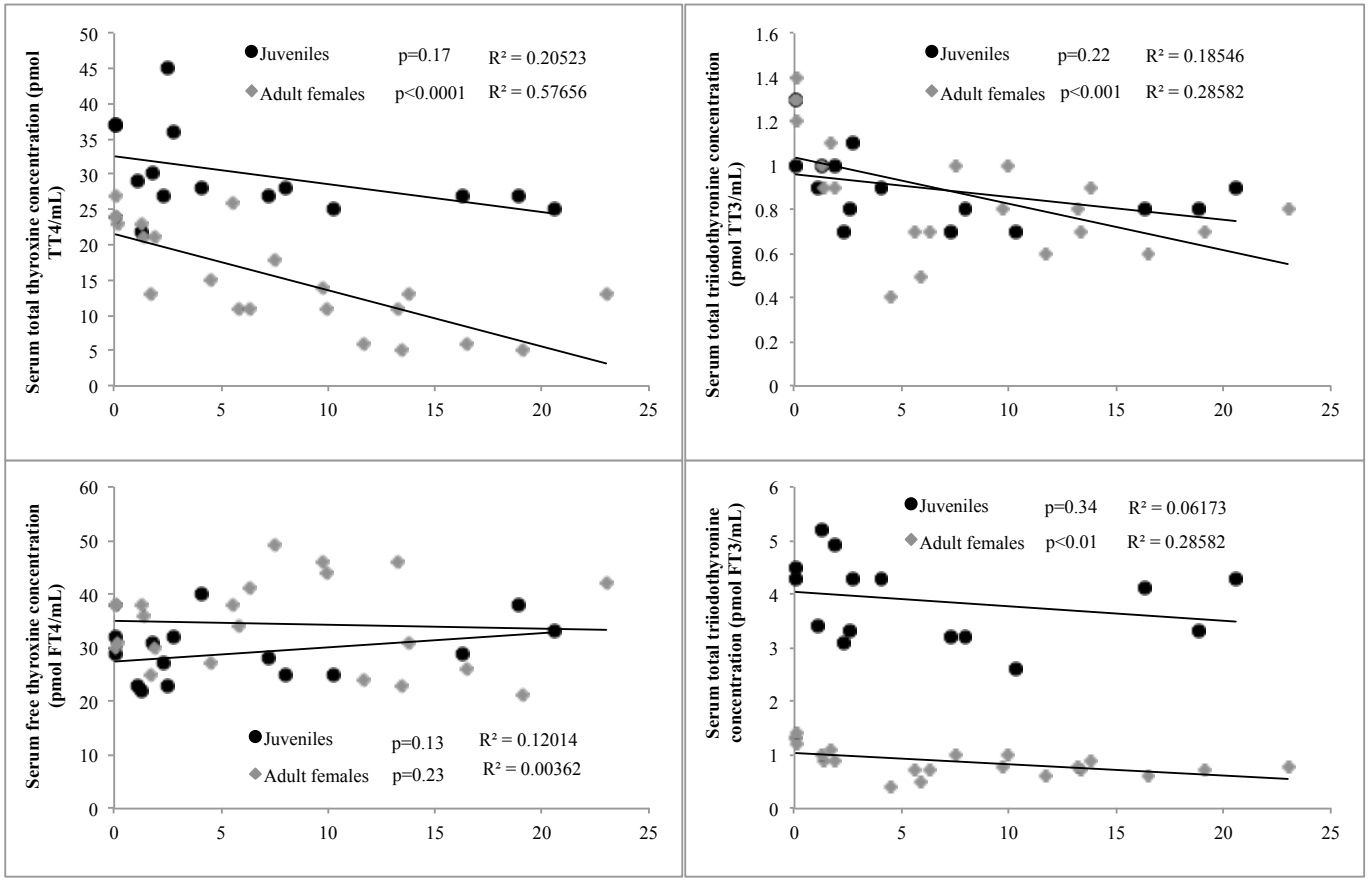


* denotes p<0.05

◆ denotes p<0.07

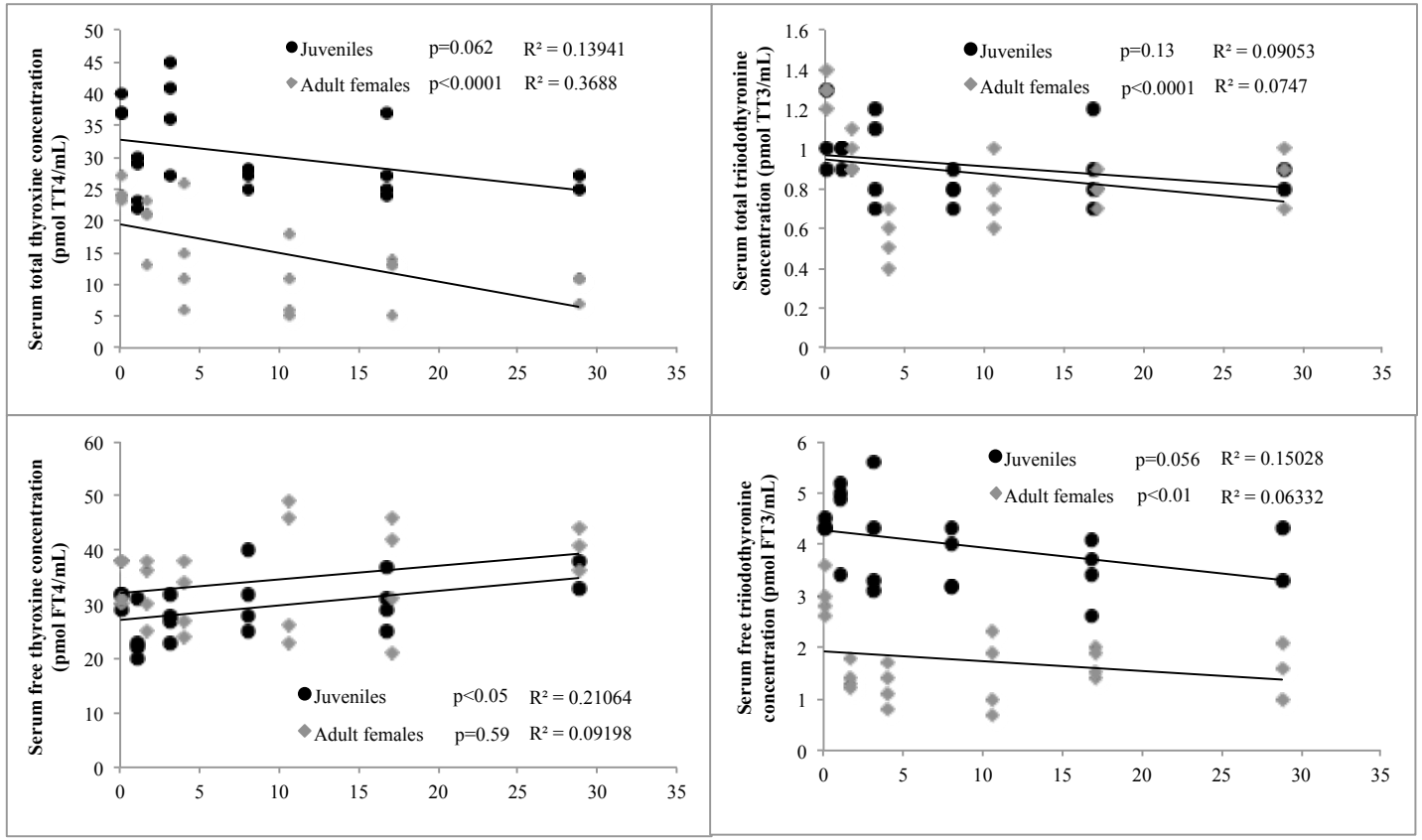
No significant decreases for juvenile TT3, FT3, FT4, or adult FT4, not shown

Figure 12. Serum thyroid hormone concentrations comparisons to the negative control in adult female and juvenile mink by treatment



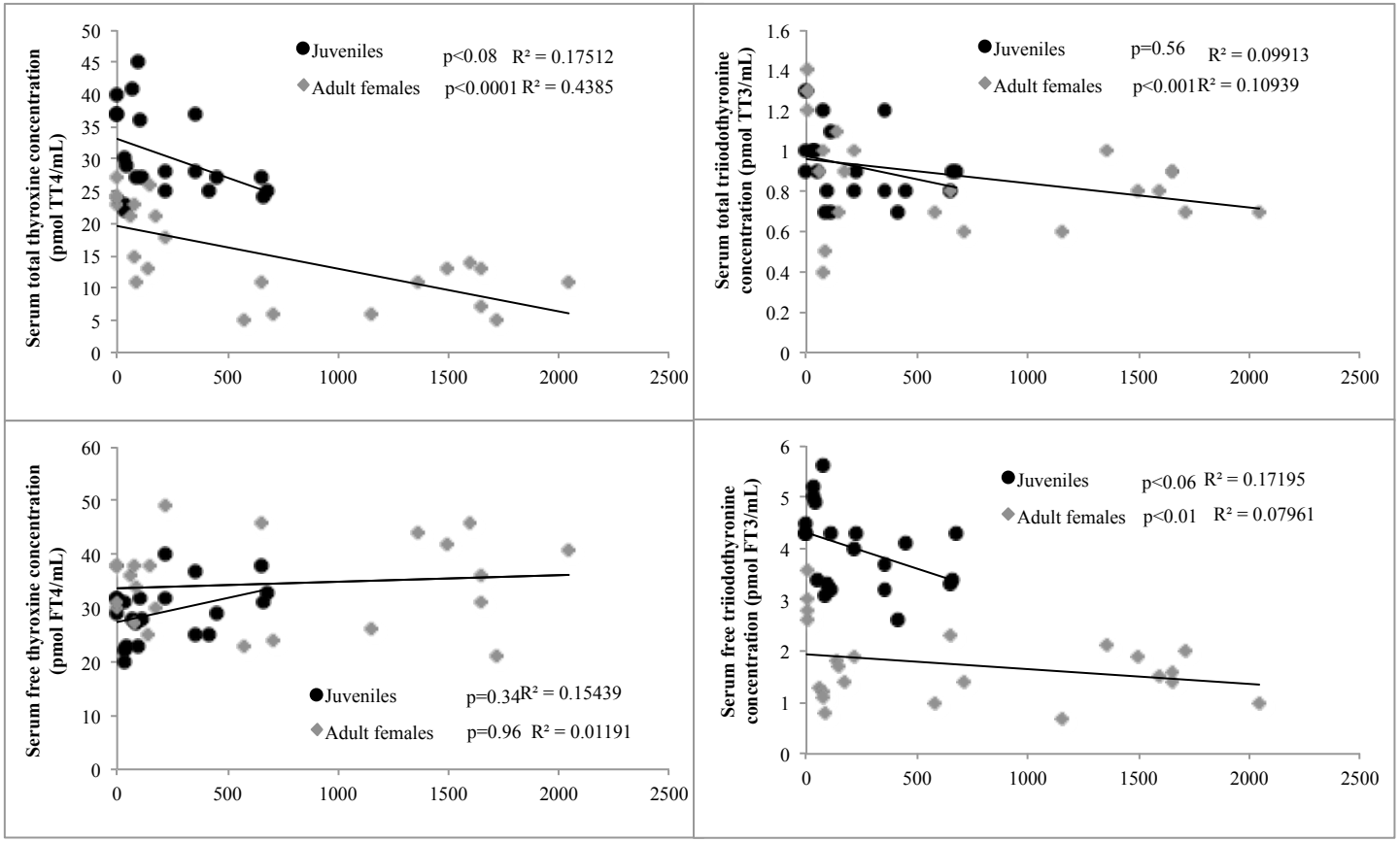
PCB: polychlorinated biphenyl
pmol: picomoles
mL: milliliter(s)
µg: microgram(s)
g: gram(s)

Figure 13. Serum thyroid hormone concentration vs. plasma PCB concentration (µg ΣPCB/mL ww) in adult female and juvenile mink



PCB: polychlorinated biphenyl
pmol: picomoles
mL: milliliter(s)
µg: microgram(s)
g: gram(s)
ww: wet weight

Figure 14. Serum thyroid hormone concentration vs. dietary PCB concentration (µg ΣPCB/g feed ww) in adult female and juvenile mink

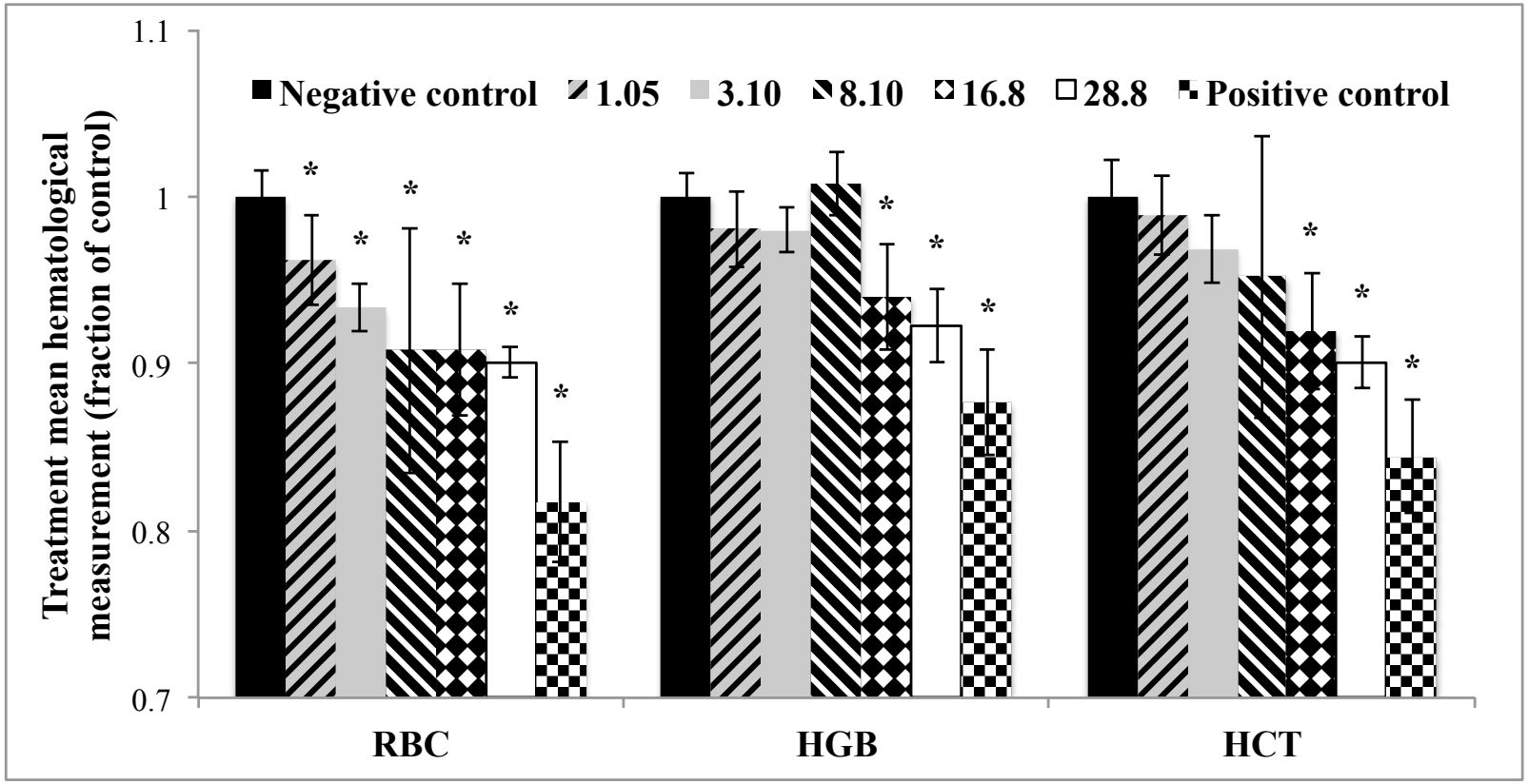


PCB: polychlorinated biphenyl
pmol: picomoles
mL: milliliter(s)
µg: microgram(s)
g: gram(s)
ww: wet weight

Figure 15. Serum thyroid hormone concentration vs. adipose PCB concentration (µg ΣPCB/g feed ww) in adult female and juvenile mink

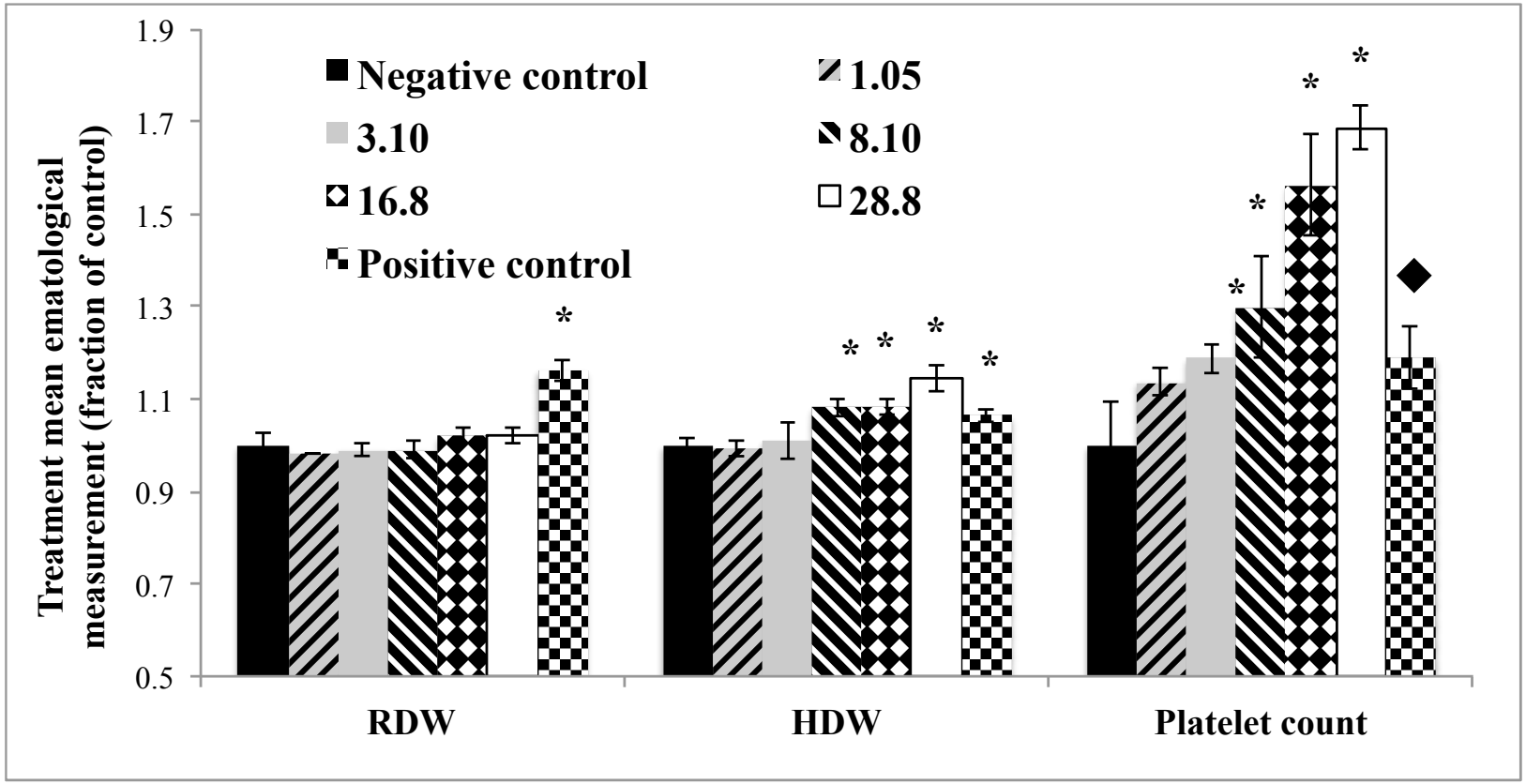
Hematology

Few hematological measurements were different from the negative control in adult female mink, although several hematological indicators of anemia were observed in juveniles exposed to Aroclor 1268 at various dietary concentrations. In adult female Aroclor 1268 treated mink, comparisons of treatment means of hematological measurements to the negative control did not reveal any dose-dependent relationships, and no adult female positive control hematological measurements were different from the negative control ($p>0.05$). In juvenile Aroclor 1268 treatments, RBC, HGB, and HCT decreased dose-dependently relative to the negative control, and treatment mean HDW and PLT measurements increased relative to the negative control ($p<0.05$). Significant dose-dependent differences in Aroclor 1268 treated juvenile mink were not observed for the remaining hematological measurements (MCV, MCH, MCHC, RDW, MPV, WBC, and TBP, $p>0.05$). In positive control juvenile mink, mean RBC, HGB, HCT, and TBP measurements were less than the negative control, and RDW and HDW were greater than the negative control ($p<0.01$). Positive control treatment mean MCV, MCH, MCHC, PLT, MPV, and WBC measurements were not different from the negative control ($p>0.05$) (Figures 16 and 17).



* Inidicates significant treatment mean differences from control ($p<0.05$)

Figure 16. Erythrocyte (RBC), hemoglobin (HGB), and hematocrit (HCT) concentrations in juvenile mink blood by treatment



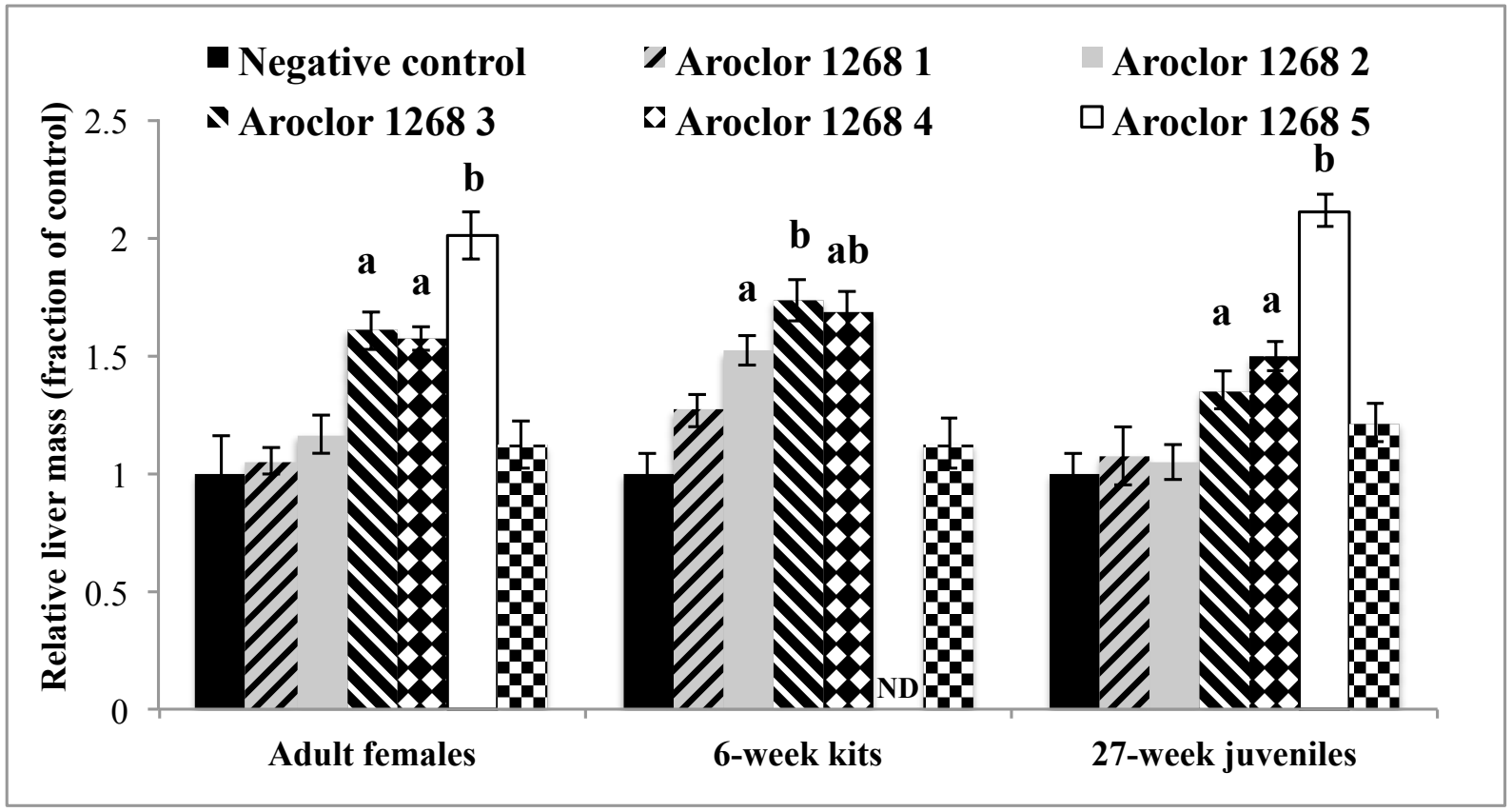
* Inidicates significant treatment mean differences from control ($p<0.05$)

◆ Indicates $p<0.06$

Figure 17. Red blood cell distribution width (RDW), hemoglobin distribution width (HDW) and platelet count measurements in juvenile mink blood by treatment

Relative organ masses

Treatment mean comparisons to the negative control and regressions with PCB exposure metrics showed that dietary Aroclor 1268 exposure affected the relative masses of few organs except for the liver. Hepatomegaly (increased liver mass) was observed in all age classes of Aroclor 1268 exposed mink. The lowest Aroclor 1268 treatment groups that had mean relative liver masses significantly greater than the negative control were 4.10 µg ΣPCB/g feed in adult female and kit mink ($p<0.05$) and 8.10 µg ΣPCB/g feed in juveniles ($p<0.01$) (Figure 18).



Letters indicate significant differences from the negative control
Groups with different letters have statistically different means
ND: No data

Figure 18. Relative liver masses by treatment for each age class

In Aroclor 1268 exposed adult female mink, relative liver masses were positively correlated with ΣPCB concentration in diet, adipose, and liver ($p<0.0001^{\lozenge}$, <0.001, and <0.0001, respectively). Relative liver masses of kit mink also were positively correlated with diet, adipose, and liver ΣPCB concentrations ($p<0.0001^{\lozenge}$, $<0.001^{\lozenge}$, and <0.001, respectively). Similar positive correlations in relative liver mass with dietary, adipose, and liver ΣPCB concentrations were also observed in juvenile mink (p<0.0001 and <0.001, <0.001, respectively). No treatment differences from the negative control were observed for relative masses of thymus, thyroid glands, adrenal glands, spleen, kidney, heart, or brain for any age class, with the exception of kidney mass in kits exposed to 17.1 µg ΣPCB/g feed (p<0.05). Similarly, the relative masses of these organs did not correlate significantly with dietary or adipose ΣPCB concentrations in all age classes (p>0.05), with the exceptions of increased adult female kidney mass vs. adipose ΣPCB concentration (p<0.05) and decreased heart, thyroid, and thymus masses vs. dietary ΣPCB concentrations in juvenile mink ($p<0.05^{ø}$, <0.01, and $p<0.05^{ø}$, respectively). The latter shared significant correlations with BCI and may have been confounded by nutritional state. Positive control mink relative masses of liver, thymus, thyroid, spleen, adrenal glands, kidney, heart, or brain were not different from the negative control for any age class (p>0.05). Decreased thymus, increased thyroid gland, and increased spleen relative masses were observed in juvenile positive control mink (p<0.0001, <0.05, and 0.001, respectively) (Table A2).

Body condition index

Adult female Aroclor 1268 treatment mean BCI values were not different from the negative control or each other at the initiation of this study ($p>0.05$). Twenty-four hours postwhelp (8 weeks after study initiation), mean treatment BCI of adult female mink in the 17.1 and 28.8 µg ΣPCB/g feed groups were significantly less than the negative control ($p<0.05$). Body condition index of adult female mink correlated negatively with dietary ΣPCB concentration but not with ΣPCB concentrations in adipose or liver ($p<0.0001$, >0.05). Treatment differences in mean BCI from the negative control did not exist in Aroclor 1268 treated juvenile mink ($p>0.05$). However, body condition in juvenile mink correlated negatively with dietary ΣPCB concentration ($p<0.05$), though not with ΣPCB concentrations in adipose or liver ($p<0.05$, >0.05) (Table 4).

DISCUSSION

Dietary exposure to Aroclor 1268 induced hypothyroidism, anemia, and hepatomegaly in mink at various concentrations, although TH concentrations and relative liver mass were not adversely affected in the positive control. Comparing effects observed in Aroclor 1268 treatments to the positive control clarifies the role of AhR mediated toxicity, given that PCB 126 is the most potent inducer of the AhR of all 209 PCBs in mink (Van den Berg et al. 2006, Beckett et al. 2008). Evidence of hypothyroidism and anemia in TBRE dolphins has been reported (Schwacke et al. 2012). Given the physiological similarities between mink and dolphins, describing these endpoints in Aroclor 1268 treated mink with comparisons to the positive control helps elucidate pathways and manifestations of Aroclor 1268 toxicity that are pertinent to TBRE bottlenose dolphins.

Cytochrome p450 enzyme induction and development of SEP jaw lesions are both mediated via the AhR pathway following exposure to dioxin-like molecules and were used as biomarkers of AhR activity (Denison and Nagy 2003, Bursian et al. 2006, Beckett et al. 2008, Haynes et al. 2009, Moore et al. 2009, Zhang et al. 2009). Results of the EROD assay and SEP jaw lesion histopathology indicate that dietary exposure to Aroclor 1268 does induce some AhR response in mink at sufficient exposures. Cytochrome p4501A1 induction in kit and juvenile mink was elevated relative to the negative control at a threshold concentration of 16.8 µg ΣPCB/g feed (or 51.4 pg ΣTEQ/g feed) (Figure 12). This finding is evidence that adverse effects observed below this threshold are likely not toxic responses acting via the AhR pathway. Jaw lesion histopathology results support this hypothesis, as well. Aroclor 1268 treatment diets induced virtually no SEP jaw lesions, despite the fact that the AhR pathway was significantly induced in the upper 2 Aroclor 1268 dose groups relative to the negative control (Table 9 and Figure 13). Development of jaw lesions in Aroclor 1268 treatments was expected to be low; however, prevalence and severity of jaw lesions in Aroclor 1268 treated mink was still less than expected if dietary ΣTEQs are used as a predictor. Specifically, the 28.8 µg ΣPCB/g feed dietary treatment had ΣTEQ concentration similar to that of the positive control, yet the 28.8 µg ΣPCB/g feed dose group individuals did not even display minor jaw lesions compared to the 100% incidence of moderate to severe lesions in the positive control. However, the sample size in the 28.8 µg ΣPCB/g feed dose group (n=2) prevents us from definitively concluding that this dose group is a no effect level. Furthermore, the capacity for Aroclor 1268 to induce CYP1A1 expression was far less than PCB 126 on a ΣPCB basis (as expected); however, CYP

expression in Aroclor 1268 treated mink was less than expected on a ΣTEQ basis as well. These unexpected results could be attributed to antagonistic interactions between congeners in the complex Aroclor 1268 mixture and the AhR as opposed to the single congener positive control, which likely encountered less interference from other PCDD, PCDF, and PCB congeners. Additionally, the TEQ scheme may overestimate the ability of some PCDD, PCDF, and non-ortho PCB congeners in Aroclor 1268 to induce the AhR pathway compared to PCB 126. Toxic Equivalent Factors can vary by species, and small differences can have order of magnitude effects on ΣTEQ concentration calculations (Giesy and Kannan 1998, 2002; Zwiernik et al. 2010). Regardless, the lack of AhR mediated responses in Aroclor 1268 treated mink, particularly in treatments below 16.8 μg ΣPCB/g feed, indicates that the proportion of non-dioxin-like PCBs to dioxin-like molecules mink were exposed to here was great enough that non-AhR mediated effects could be identified in the absence of potent AhR agonists that typically mask other toxic responses.

Multiple lines of evidence including treatment differences and negative correlations of serum TH concentrations with PCB exposure metrics in adult female and juvenile mink provided strong evidence of hypothyroidism induced by Aroclor 1268 exposure at various dietary concentrations. Total T4, TT3, and FT3 were adversely affected, although FT4 was not (Figures 14, 15, 16, and 17). A known mechanism of TH disruption by PCBs and hydroxylated metabolites involve inhibition of TH transport proteins and enzymes responsible for TH speciation (Chauhan et al. 2000, Marsili et al. 2011). Free thyroxine is secreted from thyroid glands into blood where binding to the transport protein transthyretin (TTR) occurs, forming the ligand-protein complex TT4

that is distributed to target cells for signaling. Iodothyonine deiodinase accepts T4 from TTR and enzymatically deiodinates T4 to produce T3. This is critical for TH regulation because T3 has a binding affinity for nuclear receptors more than 10-fold greater than T4; hence FT3 scarcity has a greater potential to adversely affect TH signaling (Marsili et al. 2011). Deiodinase enzymes produce the majority of T3 from T4 in cells locally, although some T3 is secreted from the thyroid for transport, while T4 is synthesized entirely in thyroid glands and is the primary transport TH. Polychlorinated biphenyls and hydroxylated metabolites may also disrupt regulation of the TH complex by inhibiting transport and deiodination of T4 to T3. Polychlorinated biphenyls and metabolites compete with T4/3 for binding sites on TTR proteins, or may induce hepatotoxicity and limit TTR production in the liver directly, both resulting in compromised TH transport (Brouwer et al. 1998, Chauhan et al. 2000). Polychlorinated biphenyls also display congener dependent competitive binding with T4 for active sites of deiodinase enzymes, with ortho-substituted PCBs showing the greatest relative potency for reducing T3 production (Chauhan et al. 2000, Debier et al. 2005, Tabuchi et al. 2006). Since the congeners characteristic of Aroclor 1268 have frequently chlorinated ortho sites, this mechanism of non-AhR mediated hypothyroidism is a plausible response.

Combinations of these TH disruption mechanisms provide possible explanations for why hypothyroidism was manifested as decreases in serum TT4, TT3, and FT3 in Aroclor 1268 exposed mink while FT4 was unaffected. This suggests that T4 production in thyroid glands did not drive the observed hypothyroidism. Rather, decreased serum TT4 and TT3 in Aroclor 1268 exposed mink suggests hypothyroidism may have resulted from disrupted TH transport, either by competitive binding of PCBs to

TTR proteins or limited hepatic production of TTR itself. Although T4 production did not appear limited, disruption of T4 transport could prevent sufficient amounts of T4 from arriving at deiodinase enzymes, negatively impacting T3 production. Aryl-hydrocarbon receptor agonistic compounds (PCB 126) have the capacity to induce hypothyroidism; however, TH measurements did not provide evidence of hypothyroidism in the positive control. This observation, coupled with the CYP1A1 enzyme induction and the SEP jaw lesion histopathology results, suggest that the kind of hypothyroidism observed in Aroclor 1268 treated mink here was mediated via a non-AhR associated pathway, or at least was not induced by the highly AhR agonistic PCB 126.

Hematology measurements were examined for indicators of microcytic, macrocytic, and normocytic anemia. Red blood cell distribution width (RDW) lends itself as a diagnostic tool for classifying how hemoglobin function is impaired. Abnormally greater or less than normal RDW is characterized as macrocytic or microcytic anemia, respectively, while normocytic anemia occurs when hemoglobin function is impaired despite normal erythrocyte volume (Tefferi 2003). All three types of anemia have been documented in response to PCB toxicity (Arnold et al. 1993a, Chu et al. 1994, Tryphonas et al. 1984). While the ecological impact of anemia of is difficult to predict, it is plausible that any PCB induced reduction in oxygen transport may have greater relevance in diving marine mammals than in mink. Cetacean and pinnipeds foraging behavior in marine ecosystems requires longer bouts of submersion at greater depths than mink, which forage in riparian and littoral zones closer to the waters' surface. Intuitively, anemia would have a relatively greater effect on mammals that rely on greater submersion times for successful foraging. Hematological indicators of anemia

were not identified in adult female mink; however, large variation in the control group limited the statistical ability to distinguish treatment differences. This large variation was likely attributed to individual hematological differences following parturition or differences in nutritional state. In juvenile mink, RBC, HGB, and HCT were decreased by Aroclor 1268 treatments and accompanied by normal RDW and decreased HDW, suggesting normocytic anemia was induced by Aroclor 1268 treatments (Figure 18). These hematological effects were mirrored in positive control juvenile mink, with the important distinction that both RDW and HDW were greater than the negative control (Figure 19). This suggests that PCB 126 also induced anemia, but of the macrocytic variety. Indicators of anemia were observed in both Aroclor 1268 treatments and the positive control; however, RDW measurements suggested there were differences in the underlying mechanism of hemoglobin dysfunction, and there may be adverse hematological effects induced by both AhR, and non-AhR agonist PCBs alike.

Hepatomegaly in Aroclor 1268 treated mink was highly dose-dependent. As a nonspecific pathology frequently documented as a toxicological response to PCB exposure, both ortho- and non-ortho-substituted PCBs can induce hepatomegaly (Safe 1994, Fernandez-Salguero et al. 1996, Schlezinger et al. 2010). However, contrary to the results of these studies, hepatomegaly was absent in positive control mink, suggesting that while mechanisms of hepatomegaly have the potential to be AhR mediated, the pathway that induced hepatomegaly in Aroclor 1268 exposed mink was not TEQ dependent. The increased liver weights of animals exposed to PCBs is typically attributed to inflammation, hypertrophy of hepatocytes through the proliferation of smooth endoplasmic reticulum, hepatic steatosis, development of lesions, or the

accumulation of necrotic hepatocytes, all of which can be confirmed histologically (Bergman et al. 1992b, Safe et al. 1994). Here, hepatomegaly was observed in all age classes of Aroclor 1268 treated mink; however, livers appeared grossly normal and were not histopathologically different from the negative control (Figure 20).

In the absence of pathological indicators of the cause of hepatomegaly in Aroclor 1268 treated mink, altered membrane permeability offers a plausible explanation. Polychlorinated biphenyls have a propensity to assimilate into cellular lipid bilayers, altering fluidity and adversely affecting cellular homeostasis; in fact, ortho-substituted PCBs may do this to a greater extent than DL-PCBs (Tan et al. 2004, Campbell et al. 2008, Reich et al. 1981). Aroclor 1268 treated mink preferentially accumulated the tri- and tetra-ortho-chlorinated PCBs 194, 201, and 206 in the liver, having higher lipid-normalized PCB concentrations in livers compared to diet, adipose tissue, and plasma (Figure 1). Given the magnitude of PCB concentrations achieved in the upper Aroclor 1268 treatments, it is possible that PCB congeners assimilated into cellular lipid membranes at a great enough proportion to alter membrane permeability, fluidity, and function. As a result, mink experiencing compromised membrane function from an elevated retention of PCBs in hepatocyte lipid membranes may have needed to generate more hepatocytes to maintain homeostasis, thus resulting in increased liver mass.

Dietary exposure to Aroclor 1268 induced hypothyroidism, anemia, and hepatomegaly in mink at various doses, while the EROD assay and SEP jaw lesion histopathology indicated the AhR pathway was only minimally induced. One of the goals of this study was to identify endpoint effects and benchmark doses associated

with Aroclor 1268 exposure in mink to provide insight on possible effects experienced by TBRE bottlenose dolphins; however, some limitations of the data should be discussed for the results to be interpreted appropriately. Literature values indicate a relatively low prevalence of PCDDs, PCDFs, and DL-PCBs in Aroclor 1268 compared to other Aroclor and environmental PCB mixtures, which prompted us to select treatment diet ΣPCB concentrations that exceeded most previous chronic PCB studies using mink of similar design (Falandysz et al. 2005, Burkhard and Lukasewycz 2008, Kannan et al. 1998, Martinez-Cored et al. 1999, Zwiernik et al. 2009). Recall that poor feed palatability was an unforeseen side effect of the high dietary concentrations administered and created a confounding factor in nutritional status. The hypothyroidism, anemia, and hepatomegaly induced by PCB toxicity are also similar effects of fasting or poor nutritional state, which occurred in adult female mink in the 17.1 and 28.8 μg ΣPCB/g feed treatments due to concentration dependent poor palatability (Eales 1998, Mustonen et al. 2005). This calls into question whether adverse effects were PCB or nutritionally driven. All statistically significant results reported here either retained their significance when individuals with low BCI values were excluded and the analysis was repeated, or effects were observed at dietary concentrations less than those associated with poor feed palatability and nutritional state. As such, it is more likely that the adverse effects observed here were primarily driven by PCB toxicity, although the extent to which nutrition contributed cannot be known. Regardless, the multiple concentration-dependent relationships observed, particularly for hypothyroidism and hepatomegaly, support the conclusion that these adverse effects were primarily toxicity driven.

Findings from this study also should be interpreted with an understanding of the differences between Aroclor 1268 neat material administered to mink and the environmental PCB mixture to which TBRE bottlenose dolphins are exposed. The congener profile of Aroclor 1268 neat material is similar to the congener profile in TBRE sediments and biota. Reductive dechlorination of PCBs by sediment bacterial communities and metabolic biotransformation throughout trophic levels can alter the composition of PCBs released into the environment from industrial operations, potentially changing the toxic potency of TBRE sediments relative to unweathered Aroclor 1268. However, the rate and extent of anaerobic dechlorination decreases with increasing degree of chlorination, and tri- and tetra-ortho-substituted PCBs, similar to those in Aroclor 1268, are resistant to weathering to the extent that total dechlorination of ortho positions is not likely (Borja et al. 2005). This suggests TBRE sediments are not likely to increase in AhR potency to a great extent compared to the original Aroclor 1268 released. Obtaining Monsanto manufactured Aroclor 1268 similar to what was used at the LCP site proved difficult. Consequently, we used Aroclor 1268 that had been synthesized more recently for research purposes. While the highly chlorinated PCB composition of the Aroclor 1268 used in this study was consistent with typical Monsanto produced Aroclor 1268, GC-HRMS analysis revealed that the PCDD, PCDF, and non-ortho substituted PCB composition was somewhat different. Polychlorinated biphenyl 126 contributes the majority of ΣTEQs in Aroclor 1268 lots produced by Monsanto, with hexa- and hepta-chlorinated furans contributing most to PCDD/PCDF derived ΣTEQs (Martinez-Cored et al. 1999, Rushneck et al. 2004, Falandysz et al. 2005, Burkhard and Lukasewycz 2008). In the Aroclor 1268 mixture administered to

mink here, 1,2,3,7,8 PeCDD contributed the majority of TEQs in diet, adipose, and liver (~65%), followed by PCB 126 (~35%). This PeCDD congener is highly AhR agonistic (TEF=1), but accounts for <1% of ΣTEQs in the TBRE environmentally weathered sediments (Kannan et al. 1998). Although certain PCDD/PCDF may have somewhat unique toxicological properties, it is uncertain how great of an effect these subtle differences in congener profiles may ultimately have on toxicity. Additionally, non-AhR active co-contaminants are also present in the TBRE, notably toxaphene and mercury, to which mink were not exposed here. It is important to consider these caveats when considering the results of this study in relation to TBRE bottlenose dolphins; however, the exposure conditions simulated using mink in this study are far more similar to that experienced by TBRE dolphins compared to any previous study. Furthermore, the range of endpoints investigated in this study provides the most comprehensive dataset describing effects of exposure to Aroclor 1268 and similar environmental mixtures to date. With this in mind, the data suggest that anemia, hypothyroidism and hepatomegaly (in mink as well as dolphins) in response to Aroclor 1268 exposure are not mediated by the AhR; hence, the TEQ approach may not be the best exposure metric to predict these types of effects.

APPENDIX

Table A2. Relative organ masses by treatment and age class

Treatment	n	Liver Mean (SD)		Thyroid Mean (SD)		Thymus Mean (SD)		Spleen Mean (SD)		Adrenals Mean (SD)		Kidney Mean (SD)		Heart Mean (SD)		Brain[c] Mean (SD)	
Adult Females																	
Negative control	9	3.430	(1.102)	0.007490	(0.002392)	0.01737	(0.01250)	30.91	(22.06)	0.01196	(0.002242)	0.7261	(0.09585)	0.7394	(0.1168)	7.528	(0.78880)
Aroclor 1268 1	8	3.602	(0.3898)	0.007691	(0.001595)	0.04921	(0.05295)	29.66	(14.95)	0.009189	(0.002437)	0.7051	(0.04560)	0.8265	(0.08210)	7.566	(0.51500)
Aroclor 1268 2	10	3.992[a]	(0.6344)	0.009268	(0.005263)	0.02033	(0.01735)	22.56	(6.589)	0.01105	(0.003041)	0.7317	(0.07461)	0.8306	(0.06957)	7.444	(0.35380)
Aroclor 1268 3	9	5.509[c]	(0.9007)	0.009021	(0.002169)	0.03173	(0.02555)	27.30	(24.49)	0.01312	(0.004326)	0.7530	(0.1390)	0.7609	(0.08736)	7.623	(0.39190)
Aroclor 1268 4	10	5.393[c]	(0.5555)	0.006006	(0.001511)	0.03125	(0.02437)	27.72	(12.01)	0.01025	(0.002186)	0.7160	(0.1255)	0.7520	(0.08469)	7.572	(0.56600)
Aroclor 1268 5	3	6.870[c]	(1.373)	0.006965	(0.003145)	0.07631[b]	(0.02478)	29.74	(4.377)	0.01245	(0.003361)	0.9222	(0.1927)	0.8279	(0.1431)	7.093	(1.31100)
Positive control	7	3.830	(0.7623)	0.008312	(0.001315)	0.03278	(0.02608)	36.36	(22.65)	0.02538	(0.044020)	0.7608	(0.1044)	0.7956	(0.06497)	7.886	(0.56200)
6-week kits																	
Negative control	10	2.582	(0.4529)	0.004660	(0.001503)	0.0748	(0.04552)	0.2220	(0.0831)	0.0079	(0.002521)	0.4585	(0.07857)	0.3520	(0.08131)	0.3585	(0.03400)
Aroclor 1268 1	10	3.273	(0.4644)	0.005972	(0.001742)	0.1243	(0.04173)	0.2958	(0.0637)	0.0080	(0.004224)	0.5058	(0.05555)	0.4300	(0.08183)	0.3120	(0.02110)
Aroclor 1268 2	10	3.915[b]	(0.4822)	0.006740	(0.001238)	0.1137	(0.03960)	0.2888	(0.0774)	0.0077	(0.002686)	0.5425[a]	(0.06864)	0.4272	(0.08297)	0.2854	(0.02194)
Aroclor 1268 3	10	4.476[c]	(0.8227)	0.006412	(0.002446)	0.08230	(0.04697)	0.2592	(0.0994)	0.0070	(0.002419)	0.4812	(0.1154)	0.3849	(0.1284)	0.3218	(0.02653)
Aroclor 1268 4	6	4.333[b]	(0.8004)	0.005813	(0.001672)	0.08801	(0.04906)	0.2488	(0.1232)	0.0113	(0.002662)	0.5237[a]	(0.1454)	0.3498	(0.1015)	0.3204	(0.02881)
Aroclor 1268 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Positive control	5	2.898	(0.6168)	0.003581	(0.000972)	0.04743	(0.02728)	0.3460	(0.0710)	0.0068	(0.002062)	0.4929	(0.08315)	0.3331	(0.08653)	0.3605	(0.03807)
27-week juveniles																	
Negative control	9	4.858	(0.8335)	0.006226	(0.001570)	0.1294	(0.04211)	0.2771	(0.0733)	0.01145	(0.002151)	0.8187	(0.8335)	0.7817	(0.08702)	0.2239	(0.01594)
Aroclor 1268 1	10	5.217	(1.288)	0.007444	(0.001421)	0.07825	(0.02652)	0.3038	(0.1954)	0.01254	(0.003447)	0.7945	(1.288)	0.8335	(0.1426)	0.2358	(0.01859)
Aroclor 1268 2	10	5.090	(0.7573)	0.006775	(0.001421)	0.1139	(0.03991)	0.2530	(0.07013)	0.01192	(0.003872)	0.7852	(0.7573)	0.7564	(0.1397)	0.2235	(0.01272)
Aroclor 1268 3	9	6.568[b]	(1.037)	0.007959	(0.001502)	0.08792	(0.03566)	0.2934	(0.1043)	0.01384	(0.004002)	0.6953	(0.1178)	0.8339	(0.1454)	0.2380	(0.01077)
Aroclor 1268 4	10	7.266[d]	(0.8900)	0.007584	(0.001498)	0.06947	(0.03325)	0.2808	(0.0611)	0.01315	(0.003226)	0.6953	(0.8900)	0.6726	(0.08576)	0.2344	(0.01601)
Aroclor 1268 5	2	10.26[d]	(1.359)	0.01030[a]	(0.002343)	0.07824	(0.01482)	0.4406	(0.0575)	0.01669	(0.003264)	0.9072	(1.359)	0.7294	(0.1046)	0.2176	(0.01164)
Positive control	9	5.888	(0.9532)	0.01326[a]	(0.013890)	0.04232[d]	(0.01601)	0.5787[c]	(0.1184)	0.01396	(0.001180)	0.8668	(0.9532)	0.7819	(0.1378)	0.2426	(0.02418)

Units are organ mass (g)/ brain mass (g)
Significant p-values indicate differences from the negative control
[a] $p<0.05$
[b] $p<0.01$
[c] $p<0.001$
[d] $p<0.0001$
[c] Normalized by body length
g: gram(s)
n: sample size
SD: standard deviation

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Study mink were continuously exposed to Aroclor 1268 in their diet with the objectives of meeting and exceeding tissue concentrations measured in TBRE bottlenose dolphins so that mammalian toxic reference values (TRVs) could be developed. The treatment diets ranged from 1.77 to 28.8 micrograms sum polychlorinated biphenyls per gram feed wet weight (µg ΣPCB/ g feed ww), or 20.3-282 µg ΣPCB/g lipid (Table 1), bracketing ΣPCB concentrations TBRE dolphin preferred prey species (8.21-113 µg ΣPCB/g lipid, Pulster et al. 2005). Adult female mink exposed to 4.01 µg ΣPCB/g ww and above had mean adipose ΣPCB concentrations that exceeded those reported for female TBRE dolphin blubber in Balmer et al. (2011) of 116 µg ΣPCB/ g lipid (Table 1).

Mink in all treatments whelped kits and percent whelp success was not different from the negative control for any treatments ($p>0.05$), although other effects on reproduction, growth, and mortality were observed. Mean litter size was less than the negative control for the 10.6 µg ΣPCB/g ww treatment ($p<0.05$), but not for any other treatments. Kit mass at whelp, 3 weeks, and 6 weeks of age, as well as juvenile body mass and length at 27 weeks of age were adversely affected by dietary Aroclor 1268 exposure around 17.1 µg ΣPCB/g ww. Kit mortality in the 28.8 µg ΣPCB/g ww treatment was greater than the negative control. Poor palatability of feed, as well as an elevated occurrence of infanticide confounded growth and reproductive endpoints (see Chapter 1 Discussion for details).

Additional goals of this study included identifying individual health endpoints adversely affected by Aroclor 1268 and describing the underlying role of the aryl hydrocarbon receptor (AhR) pathway in manifesting toxicity. This was done through the

comparison of a highly AhR agonistic positive control (PCB 126) treatment to Aroclor 1268 treatments, as well as by using an EROD assay and the presence of squamous epithelial proliferation (SEP) jaw lesions as qualitative indicators of AhR activity. The results of the ethoxy resorufin-o-deethylase (EROD) assay indicate that cytochrome p450 1A1 (CYP1A1) was expressed to a greater extent in the positive control than any Aroclor 1268 treatment. Furthermore, the EROD assay results indicate that CYP1A1 activity in Aroclor 1268 treatments was only greater than the negative control at or above dietary treatments of 17.1 µg ΣPCB/ g ww in kit and juvenile mink (Figure 12). Moderate to severe SEP jaw lesions were present in 100% of positive control mink, while jaw lesions were only observed in 2 of 41 Aroclor 1268 treated mink and were characterized as mild (Table 9 and Figure 13). The EROD assay and jaw lesion histopathology results indicate that Aroclor 1268 toxicity is mediated through a pathway additional to the AhR. Hepatomegaly, hypothyroidism, and normocytic anemia are examples of endpoint effects that were likely mediated by a non-AhR pathway because they were present in Aroclor 1268 treated mink that did not have elevated CYP1A1 activity or show signs of SEP jaw lesions, and these endpoint effects were not observed in positive control mink (see Chapter 2 Discussion for details).

Toxicity benchmarks were identified for adversely affected endpoints. These benchmarks include no observed adverse effect concentrations (NOAECs) and lowest observed adverse effect concentrations (LOAECs), as well as effect concentration (EC) estimates for a subset of growth endpoints, and lethal concentration (LCp) estimates (LC) for kit mortality at 6 weeks of age. These values are summarized in Tables 6 and 7, and Figures 4, 5, 6, 7, 8, and 9). The most sensitive growth/reproductive dietary

LOAEC was kit mass at 3 weeks of age (10.6 µg ΣPCB/ g ww). Individual health measurements including hypothyroidism and anemia were more sensitive than growth or reproductive endpoints, with dietary LOAECs of 1.05 µg ΣPCB/ g ww for total thyroxine (TT4), total triiodothyronine (TT3), and free triiodothyronine hypothyroidism (FT3), and decreased erythrocyte count (RBC) in juvenile mink (Figures 14 and 18).

Comparisons of kit mortality in this study to previous PCB toxicity studies using mink reveals that lethal concentrations of Aroclor 1268 appear to be 1-2 orders of magnitude greater than Aroclors 1254, 1248, and 1242, as well as environmental mixtures of PCBs in Great Lakes fish species (Bleavins et al. 1980, Aulerich et al. 1987, Heaton et al. 1995, Bursian et al. 2006, Bursian et al. 2013). It should be noted, however, that differences in the design of this study and the PCB composition used limit direct comparability to these previous studies. Comparisons of mink to bottlenose dolphins are further hindered by species differences in reproductive strategy, PCB metabolism, elimination, and partitioning, all having an overall effect on species sensitivity. It is clear that adult female mink in this study experienced exposure to Aroclor 1268 that exceeded what has been reported in TBRE bottlenose dolphins, yet reproductive failure was not induced. However, adversely affected offspring growth and individual health endpoints in mink occurred at exposures more similar to those observed in TBRE dolphins. The apparent low AhR activity of Aroclor 1268 allowed the non-AhR mediated adversely affected individual health measurements of hepatomegaly, hypothyroidism, and normocytic anemia to be elucidated in mink without being masked by lethal effects of the typically more AhR potent coplanar PCBs, polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans

(PCDFs), and complex toxicity pathways these congeners induce. These findings have implications for describing the toxicities of PCB mixtures that lack substantial PCDD, PCDF, or coplanar PCB composition. This study highlights the notion that competitive inhibition by multiple PCB congeners in complex mixtures, coupled with the apparent presence of non-AhR mediated toxicity, and questions the applicability of both ΣPCB and sum 2,3,7,8-TCDD equivalent (ΣTEQ) approaches for describing dietary or tissue based exposures. Ultimately, the importance of understanding the unique toxicological properties of PCB congeners is of great importance when estimating risk posed to wildlife by environmental PCB mixtures.

Research investigating the effects of PCB exposure has been largely focused on coplanar PCBs that act through the AhR pathway, despite the fact that environmental PCB contamination of sediments are always complex mixtures that contain coplanar and non-coplanar congeners of varying relative abundances. Although coplanar PCBs tend to induce adverse effects at lesser concentrations, both AhR and non-AhR mediated effects likely occur simultaneously. There is a need for future research that increases the understanding of concurring mechanisms of toxicity. The existing TEQ measurement scheme attempts to describe AhR mediated toxicity in a manner that accounts for the relative potencies of individual congeners, although this approach assumes that all congener effects are additive, which is not necessarily a valid assumption. Identifying congener specific relative potencies for each outcome and possibly synergistic/antagonistic relationships should be a priority. Additionally, effort should put towards developing similar schemes for measuring PCB exposures that induce non-AhR mediated toxicity pathways that also attempt to describe synergism

and antagonism. To take this a step further, attempts should be made to understand synergistic and antagonistic effects of interactions between coplanar and non-coplanar PCBs. Although non-coplanar PCBs have little to no affinity for the AhR, it's plausible that non-coplanar PCBs may inhibit coplanar PCBs from interacting with the AhR through physical interference when non-coplanar PCBs are present at high enough concentrations. This conceptual framework of PCB interactions suggests that measuring the ratio of non-coplanar PCBs to coplanar PCBs in addition to the total concentration may provide valuable information for predicting toxicity.

In the future, the ability of risk assessors to interpret tissue concentrations and predict adverse effects of Aroclor 1268 exposure would be improved by identifying adverse outcome pathways for the non-AhR mediated effects of normocytic anemia, hypothyroidism, and hepatomegaly described in this thesis. Using in-vitro assays that expose cultures of thyroid epithelial (follicular) cells, hematopoietic cells from bone marrow, and hepatocytes to Aroclor 1268 may illuminate specific dysfunctions that ultimately elicited the results observed in this experiment. Using microarrays to describe changes in gene transcription may further elucidate these adverse outcome pathways. The large number of PCB congeners and the differences in toxicological responses they can induce present tremendous challenges towards attaining a comprehensive understanding to PCB toxicity. While the understanding of PCB ecotoxicology has advanced substantially, large data gaps describing congener interactions and concurring mechanisms of toxicity still exist.

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