Bioaccumulation and physiological responses of the turtle *Chelydra serpentina* exposed to polychlorinated biphenyls during early life stages

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Abstract

Despite the North American production ban of polychlorinated biphenyls (PCBs), PCBs are ubiquitous in the environment and in wildlife tissues. *Chelydra serpentina serpentina* (common snapping turtle) have been used as environmental indicators of PCB pollution upwards of 40 years given their high site fidelity and high trophic position. Despite their long use as indicators of PCB contamination, the effects of PCBs in reptiles remain largely unknown. In this study, we performed two experiments to assess i) bioaccumulation and ii) toxicity of PCBs to 1-month-old *C. s. serpentina*, to aid in interpretation of PCB burdens. Food pellets were spiked at an environmentally relevant concentration (0.45 µg/g) of the PCB mixture Aroclor 1254 to model hepatic bioaccumulation and depuration, through feeding, for 31 days and clean food for 50 days, respectively. No significant differences in PCB concentrations were observed in liver tissue over the course of the experiment, suggesting that juvenile turtles can likely metabolize low environmentally occurring concentrations of PCBs. Additionally, a dose-response experiment, performed to determine hepatic toxicity and bioaccumulation in juvenile *C. s. serpentina,* showed a 1.8-fold increase in hepatic expression of *cyp1a* when fed A1254-spiked pellets (12.7 µg/g; range 0 - 12.7 µg/g). This gene induction correlates with the significant increase of group 3 PCB congeners measured in the turtle liver, which are known to be metabolized by CYP1A. This study indicates that *C. s. serpentina* may be a good environmental indicator for PCBs while more research is needed as body burdens are increased in wild *C. s. serpentina*.

1. Introduction

Although polychlorinated biphenyl (PCB) production was banned in 1977 in North America (Kimbrough, 1987), PCBs are still one of the more abundant organic contaminant groups found in wildlife tissues (including in turtles) (Safe, 1994; de Solla et al., 2016; Barraza et al. 2020). The environmental ubiquity of PCBs is partly due to their lipophilic properties and long half-lives, as PCBs can reside in soil for upwards of 24 years, depending on the congener (Ayris and Harrad, 1999). PCBs induce oxidative stress, which may result in cellular damage (Winston and Di Giulio, 1991; Glauert et al., 2008). Following PCB exposure, a frequent response is to increase the activity of metabolism and detoxification enzymes to metabolize and increase elimination or to reduce toxicity. For example, the activity of cytochrome P450 (CYP), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) genes was altered in fish, frogs, and birds after exposure to PCBs (Palace et al., 1996; Jin, et al., 2001; Vega-López et al., 2007). Cytochrome P450 1A and 2B (CYP1A and CYP2B, respectively) are two of the enzymes responsible for PCB hydroxylation (Letcher et al., 2000). In addition, PCBs are known endocrine disrupting chemicals (EDCs) and can disrupt normal growth and development by interfering with the hypothalamus-pituitary-thyroid gland (HPT) axis and disrupt sexual development and reproduction through the hypothalamus-pituitary-gonadal (HPG) axis (Crain, et al., 1998; Willingham, 2001; Brown et al., 2002; Cai et al., 2011; Holliday et al., 2009). Growth rates were also reduced following PCB exposures in turtles and birds (Bishop et al., 1994; Hoffman et al., 1996; Holliday et al., 2009).

Despite the relatively rich literatures on PCBs, their effects in reptiles is relatively depauperate. Although there have been studies showing increased deformities of hatchlings or reduced hatching success of turtles from populations in contaminated sites (Bishop et al., 1991; de Solla

et al., 2008), causal links to PCBs specifically have been difficult to make, given that the turtles are exposed to complex mixtures of contaminants. PCB concentrations in adult red-eared sliders (*Trachemys scripta elegans*) were correlated to hematological and immune function in turtle captured from turtles caught in ponds near a Gaseous Diffusion Plant (Yu et al., 2012). Similarly, Rousselet et al (2017) found that PCBs increased eosinophil phagocytosis and decreased natural killer white blood cell activity in blood samples of *Caretta caretta* (loggerhead sea turtles) exposed to PCBs. Dehydroretinol and retinol concentrations were inversely proportional to PCBs in adult *C. s. serpentina* sampled among several wetlands with a gradient in PCB contamination, in the lower Great Lakes (Letcher et al., 2015). Dietary exposure of PCBs at ~6 µg/g ww to juvenile *Chelydra serpentina serpentina* (common snapping turtle) caused reduced growth rates and induced mortality in turtles previously exposed to high levels of PCBs through maternal transfer, but not to juveniles that were not previously exposed (Eisenriech et al., 2009).

C. s. serpentina have been used as environmental monitors for almost 40 years to assess their health or body burdens in contaminated environments (e.g., Helwig & Hora, 1983; Eisenreich et al., 2009; Hughes et al., 2019) and temporal or spatial trends of environmental contaminant levels (de Solla et al., 2016; Lu et al., 2019). *C. s. serpentina* are opportunistic omnivores, feeding on aquatic plants, invertebrates, fish, and even young turtles. Adult snapping turtles are typically at the high end of the food chain, thus likely to biomagnify PCB levels seen at lower levels of the food chain. In addition, turtle eggs contain a lipid rich yolk, which can sequester PCBs (de Solla et al., 2007), that are deposited through maternal transfer from the laying female. As an example, most PCB burdens in eggs of *C. s. serpentina* varied between approximately 0.002 to 3.7 µg/g (ww) in the Great Lakes (de Solla et al., 2007; Dabrowska et al., 2006). Higher concentrations in *C. s. serpentina* eggs have been observed in other contaminated areas, such as

101 up to 12.1 µg/g in the Hudson River (NY; Kelley et al. 2008), and up to 736 µg/g PCBs from
102 Akwesasne (NY) (de Solla et al., 2001), both of which are downstream of known PCB sources.
103 The toxicity of PCBs has been investigated in almost every vertebrate taxonomic group;
104 however, reptiles have had relatively little attention in terms of effects of PCBs, specifically at
105 the juvenile stage (Adams et al., 2016). Relating body burdens in wild turtles to exposures or
106 body burdens sufficiently high to elicit biological responses is currently very limited. Therefore,
107 this study assessed the levels of PCB exposure capable of causing morphological and genetic
108 expression responses in juvenile *C. s. serpentina* through chronic dietary PCB exposure. We
109 used a dosing regimen that would give similar PCB exposures to the turtles as they would have
110 gotten through maternal transfer (e.g. Bishop et al. 1995), leading to body burdens in the fed
111 turtles comparable to those expected of hatchlings from PCB contaminated areas. In our first
112 experiment, our objective was to assess bioaccumulation and depuration rates of turtles exposed
113 to PCBs through diet over a period of 80 days. We predicted that PCBs will accumulate, in a
114 dose dependent response, in turtle livers and that depuration rates would be proportional to lipid
115 solubility of individual PCBs. For our second experiment, our objective was to assess the toxicity
116 of PCBs in diet to turtles, in a geometric series of concentrations. We predicted that
117 transcriptional changes of targeted genes will be consistent with an increase in detoxification,
118 metabolism, oxidative stress, and endocrine disruption as a response to PCB exposure. As turtles
119 have a relatively high site fidelity (Muñoz and Vermeiren 2018), *C. s. serpentina* could serve as
120 good bioindicators of their site contamination through dietary exposure.

121 **2. Materials and methods**

122 **2.1 Animals**

123 Eggs were collected from recently laid *C. s. serpentina* nests located adjacent to Long Point
124 Provincial Park (ON, Canada) on June 11th, 2014, a site that has relatively low known PCB
125 burdens in *C. s. serpentina* eggs (0.02 – 0.16 µg/g; de Solla, unpublished data). Nests were
126 located by visual inspection, and eggs were excavated and placed immediately in 4.7-L plastic
127 bins containing vermiculite and water mixture (1:1) to maintain adequate hydration. A total of 10
128 clutches were collected. Eggs (n = 170) were brought to the Canada Centre for Inland Waters
129 (CCIW) at Environment and Climate Change Canada (ECCC, Burlington, ON, CA) for
130 incubation in an environmental chamber at 24 °C to produce a 1:1 mixed ratio of males to
131 females. The environmental chamber was held at approximately 75% humidity, and eggs were
132 kept in 1:1 vermiculite and water mixture, which was kept moist by spraying with spring water.
133 Turtles hatched between August 28th and September 5th, 2014. Normally they have a residual
134 yolk sac, and they overwinter until spring. We reared the hatchlings unfed until they absorbed
135 their yolk sac. Hatchlings were then brought to Queen's University Animal Care Facility,
136 Kingston, ON, CA on October 9th, 2014. The room temperature was kept at 23 ºC ± 3 ºC.
137 Animals were fed Martin PROFISHENT™ trout chow (Elmira, ON, CA), *ad libitum* for one
138 month as an acclimatization period before beginning the experiment. During the exposures, each
139 hatchling was fed five pellets twice a week. Animal care protocols followed the guidelines from
140 the Animal Care Committee of Queen's University (Kingston, ON, CA) and the Canadian
141 Council of Animal Care.

142 **2.2 Pellet dosing**

143 The trout chow was dosed with the commercial PCB mixture Aroclor 1254 (A1254), due to its
144 relatively high production and its prevalence in the environment and in turtle eggs (e.g. de Solla
145 et al., 2007), at CCIW using a rotary evaporator (Buchi Vacobox B-177, Taylor Scientific St.

146 Louis, MO, USA). A stock solution of 0.2 mg/L A1254 (100%; CAS 11097-69-1; Sigma
147 Aldrich, St. Louis, MO, USA) was made by dissolving A1254 into acetone (99.7% pure;
148 Georgetown, ON). Trout chow was placed into the bottom flask and the appropriate volume of
149 A1254 stock solution was added to acetone such that the final acetone volume was of 100 mL.
150 The control food was treated the same way and 100 mL of pure acetone was added to the flask.
151 Thereafter, mixtures were gently mixed and placed under the fumehood for 30 min. The food
152 mixture was placed in a rotary evaporator for about 1 h until dry, and subsequently placed on
153 aluminum foil in a fume hood for 24 h. The food was then stored in plastic containers and kept
154 frozen at -20 ºC. PCB concentrations were measured by GC-MS in food and are reported in
155 Section 3.2.2.

156 **2.3 Tissue collection**

157 Turtles from both experiments were sacrificed by decapitation using sharp scissors, to ensure one
158 clean cut, cleaned with diethylpyrocarbonate (DEPC) water and 10% hydrogen peroxide. The
159 brain, liver, and GMC (gonad-mesonephros complex) from all turtles were sampled on each
160 sample day for the toxicokinetics study (days 1, 2, 5, 8, 12, 17, 25, and 31 during the
161 bioaccumulation period and on days 32, 33, 34, 35, 37, 40, 44, 49, 54, 61, 71, and 81 during the
162 depuration period) and the end of the exposure for the toxicity exposure and immediately stored
163 at -80 ºC until further analysis. Tissues were weighed to calculate somatic indices and livers
164 were collected to assess differential gene expression.

165 **2.4 Toxicokinetics experiment**

166 A total of 100 hatchlings were randomly selected as to avoid clutch and maternal effects, to
167 model the accumulation and depuration rates of A1254 within their livers. Animals were
168 individually housed in 2.2-L plastic containers with approximately 250 mL of water that was

169 sloped to allow the animals to submerge but also ensured a dry area to bask. Turtles were fed
170 five A1254-spiked pellets (nominal dose of 0.5 μg/g, ww) biweekly for 31 d (bioaccumulation
171 period). The dosage was chosen based on the PCB concentrations measured in invertebrates and
172 small fish that would form the diet of juvenile *C. s. serpentina* and to minimize growth (Zaranko
173 et al., 1997; Walters et al., 2008). Following the 31-d exposure, turtles were fed clean pellets
174 biweekly for an additional 50 d (depuration period). PCB liver concentrations on day 1 were
175 used as a negative control.

176 **2.5 Toxicity experiment**

177 A chronic exposure to A1254 was performed using 70 *C. s. serpentina* hatchlings. Turtles (14
178 per treatment) were individually housed in 2.2-L plastic containers with identical environmental
179 conditions as the toxicokinetics experiment. Turtles were randomly but evenly spread amongst
180 the different treatments to avoid clutch or maternal effects. Turtles were exposed through feeding
181 to a range of concentrations of A1254-spiked pellets (nominal doses of 0, 0.1, 0.5, 2.3, and 12.5
182 μg/g, ww) for 81 days. Each hatchling per treatment group was fed five pellets biweekly. Turtles
183 exposed to a nominal dose of 0 μg/g, ww were used as negative controls.

184 **2.6 Morphometrics**

185 Several morphometric measurements were taken at each sampling day, after the animal was
186 sacrificed, during the toxicokinetics experiment and at the end of the toxicity experiment and to
187 determine the effects of A1254 in juvenile turtles. Turtles were chosen on a random basis for the
188 toxicokinetics experiment. Total body and carapace length, body, brain, liver, and GMC masses
189 were recorded for all turtle samples. Gonadal somatic index (GSI) and hepatic somatic index
190 (HSI) were calculated by percentage of GSI or liver mass/body mass.

191 **2.7 Gas chromatography with mass selective detection (GC-MSD)**

192 *C. s. serpentina* livers (n = 7/treatment for toxicity experiments and n=3-4 per sample day for
193 toxicokinetics experiment) were analyzed for PCB congener content at the National Wildlife
194 Research Centre, ECCC (Ottawa, ON, CA). PCBs from liver and food samples were extracted
195 using dichloromethane:hexane (1:1 v/v). Gel permeation chromatography was used to remove
196 lipids and samples were cleaned by column chromatography. Samples were analyzed from a
197 single florisil fraction for PCBs by gas chromatography (GC; Agilent 6890N; Agilent
198 Technologies, CA, USA) using mass selective detection (MSD; Agilent 6890N) in selected ion
199 monitoring (SIM) mode. Thirty-seven PCB congeners were analyzed, as identified by the
200 International Union of Pure and Applied Chemistry (IUPAC): 18/17, 31/28, 33, 44, 49, 52, 70,
201 74, 87, 95, 99, 101, 105, 110, 118, 128, 138, 149, 151, 153, 156, 158, 170, 171, 177, 180, 183,
202 187, 194, 195, 199, 205, 206, 208, and 209.
203 The method detection limits (MDLs) for quantification and limits of detection (LODs) for the
204 target compounds are listed in Table S1. Analytes with signal-to-noise ratios less than three were
205 reported as below LODs. The MDL was defined as the concentration yielding a signal to noise
206 ratio of 10. The recoveries for internal standards were determined by the external standard
207 method. The mean percent recovery of four spiked quality control samples (NIST 1947, Lake
208 Michigan Fish Homogenate) was 84.4% (54.1%–163.5%). The mean percent recovery of
209 internal standards (PCB 28, 52, 118, 153, 180, and 194) was 74.5% (38.9%–146.5%; Table S.2).
210 **2.8 RNA isolation**
211 Total RNA was sampled from livers using TRIzol solution (TRIzol® Reagent, Life
212 Technologies, Burlington, ON, CA) as recommended by the manufacturer. Briefly, 4M lithium
213 chloride (LiCl, Sigma Aldrich) was added and the sample vortexed until the pellet dissolved and
214 re-pelleted by centrifugation. The supernatant was discarded and the pellet was washed two more

215 times with LiCl. The pellet was washed with 80% ethanol (Commercial Alcohols, Inc.,
216 Brampton, ON, CA), air-dried, and the pellet resuspended in 20 µL of nuclease-free water. The
217 samples were measured for total RNA concentration and purity using a NanoDrop-2000
218 spectrophotometer (Fisher Scientific, Ottawa, ON, CA) and stored at -80 ºC. DNA was removed
219 from the samples using DNase I treatment with the Promega RQ1 RNase-Free DNase kit (Fisher
220 Scientific) following the manufacturer's protocol. Random primers, provided with the kit, were
221 used to convert RNA to 1 µg of complementary DNA (cDNA) using Promega GoScript™
222 Reverse Transcription System Kit (Madison, WI, USA) as per manufacturer's protocol. Samples
223 were kept at -20 ºC until further use.

224 **2.9 Gene expression**

225 Transcriptomic alterations were assessed by measuring the expression of 14 genes (*i.e., thrα*,
226 *thrβ*, *dio2*, *dio3*, *esr1*, *ar*, *ahr, arnt, cyp1a*, *cyp2b5*, *gpx1*, *cat*, *sod1*, and *hsp7*mRNA*;* refer to
227 Table S1 for gene names) in 6-10 *C. s. serpentina* liver. Variation in sample size (n) (6-10) are
228 due to variability in successful extractions of RNA and from the removal of outliers. Specific
229 primer sets for quantitative real-time polymerase chain reaction (qPCR) were designed based on
230 *Chelonia mydas* (green sea turtle) or obtained from Rhen et al. (2007). The specificity of each
231 primer set was confirmed with the pGEM®-T vector cloning system (Promega, Madison, WI,
232 USA). DNA sequencing of amplicons were obtained through Robarts Research Institute
233 (London, ON, CA). qPCR analysis was performed on the Agilent Mx3005P Real-Time PCR
234 instrument (Agilent Technologies, Inc., Santa Clara, CA, USA) using the Promega GoTaq Bryt®
235 Green qPCR Master Mix (2X; Fisher Scientific). For each qPCR assay MIQE guidelines (Bustin
236 et al., 2009) were followed with a negative template control and a negative reverse transcriptase
237 control were included to ensure no contamination. A standard curve was prepared through serial

dilution (1:4) starting at 50 ng. All samples, controls, and the standard curve were run in duplicate. Efficiencies were between 91 and 124%, and coefficients of determination (R^2) were above 0.97. Gene expression was normalized to the ribosomal protein L8 (*rpl8*) and ornithine decarboxylase (*odc*). Gene expression changes was reported as fold changes relative to the control.

2.10 Data analysis

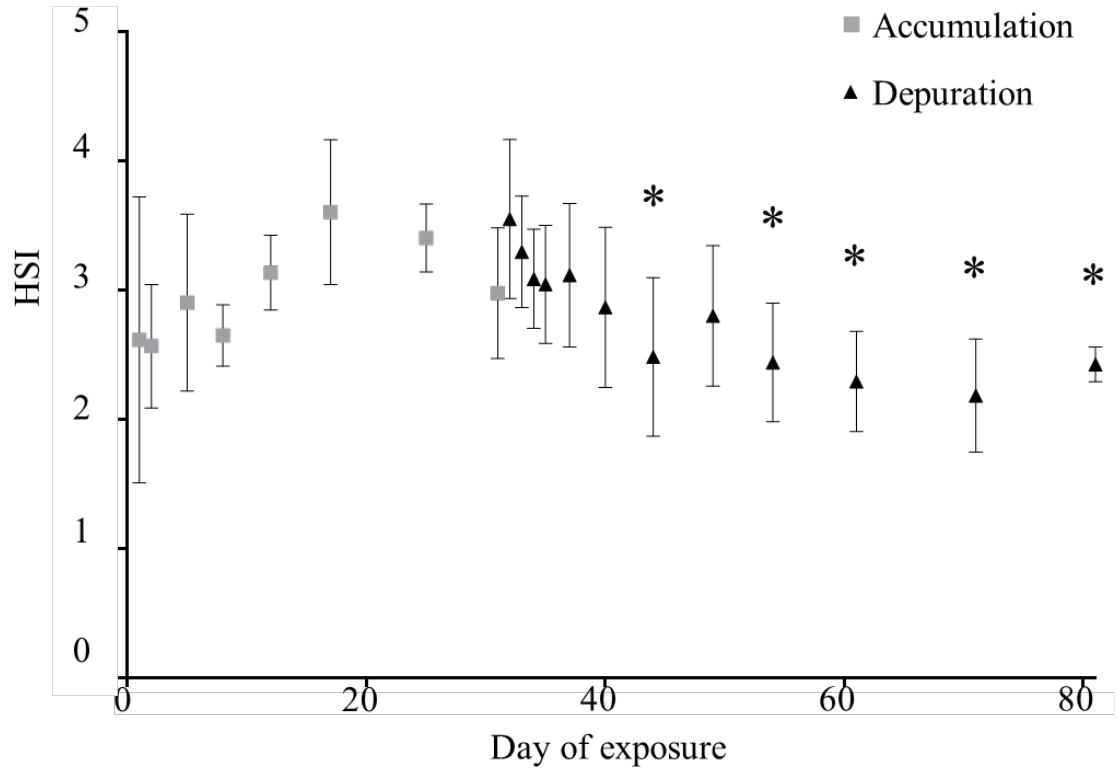
Individual gene expression levels were considered outliers if they were outside the 1.5X interquartile range and were removed from datasets prior to analyzing the gene expression data. Log_{10} and square root transformations were calculated on gene expression data that were not normally distributed. Mean sum PCB concentrations, PCB congener patterns, and expression of all genes were compared among treatments using ANOVA (analysis of variance) and Tukey Honest Significant Difference (HSD) multiple comparison tests. Linear regression was used to assess relationships between PCBs and response variables. Differences were considered significant if *p*-values were ≤ 0.05. PCB congeners were divided into four groups depending on which of CYP1A and CYP2B are responsible for the congener's metabolism. Group 1 congeners are mostly non-metabolized, do not contain meta-para (*m*,*p*) or ortho-meta (*o*,*m*) vicinal H-atoms, and have 5-10 chlorine atoms; group 2 are metabolized by CYP2B, contain only *m*,*p* vicinal H-atoms, and have 4-9 chlorine atoms; group 3 is metabolized by CYP1A, has only *o*,*m* vicinal H-atoms, and has 3-7 chlorine atoms; and finally, group 4 congeners can be metabolized by both CYP1A and CYP2B, contain *m*,*p* and *o*,*m* vicinal H-atoms, and have 2-7 chlorine atoms (Kannan et al., 1995). We compared the proportion of PCBs from each group to the sum PCBs using ANOVA, and we assessed the relationship between each PCB group and CYP1A and CYP2B using regression. Concentrations of PCB 101, 105, 118, 128, 138, 153, 170, and 180

only (as they were complete data sets, with no data points below LOD) were converted to proportions of Σ_{37}PCBs measured and used for principal component analysis (PCA), to assess changes in the PCB profiles in turtle liver among treatments. ANOVAs and regressions were performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA, USA). PCA was analyzed by R (R Core Team, 2013). 3D Visualization Using OpenGL (rgl; Adler, et al., version 0.95.1441, 2016) and Tests for Normality (nortest; Gross and Ligges, version 1.0-4, 2015) packages were used in R.

3. Results

3.1 Morphometric measurements

Mean animal weights on day 1 were 12.15 ±2.01 g and were 11.43 ± 1.61 g on day 81 for the toxicokinetics experiment. Liver mass decreased by 39.8% at the end of depuration ($R^2 = 0.32$, $F_{[1,58]} = 31.67$, $p < 0.001$) compared to controls (Figure S1). In addition, during the toxicokinetics exposure, HSI decreased by 35.1% (Figure 1; $R^2 = 0.34$, $F_{[1,58]} = 29.28$, $p < 0.0001$) during the depuration period. No significant differences were found among treatments groups in total body length, carapace length, GMC mass, and/or GSI following exposure. No significant changes were observed following the toxicity experiment (Table S2).



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278 **Figure 1**. HSI (hepatic somatic index) of juvenile *C. s. serpentina* exposed during a 31-d dietary A1254
279 exposure followed by a 50-d depuration period. The first 31 d correspond to the accumulation period
280 (pellets containing 0.45 µg Σ_{37}PCBs/g) and beyond 31 d corresponds to the depuration period (clean
281 pellets). Asterisks (*) show statistical difference compared to day 32 (HSI 3.55 ± 0.6; first day of
282 depuration) determined by one-way ANOVA followed by Tukey HSD for multiple comparisons. Data (n
283 = 5) are presented in mean ± SD.

284

285 **3.2 PCB analysis and liver accumulation factors**

286 **3.2.1 Toxicokinetics experiment**

287 The toxicokinetics study used pellets spiked with 0.45 µg Σ_{37}PCBs/g during the accumulation
288 period and clean pellets (0 µg Σ_{37}PCBs/g) for the depuration period. Liver concentrations (n = 3-
289 4) were measured at each sampling day to model accumulation and depuration of A1254 (Figure
290 S2). Mean concentration of PCBs in the liver were measured at 0.56 µg Σ_{37}PCBs/g during the
291 exposure, but no significant differences were observed between the treated juvenile turtles

compared to day one at any given time point (Figure S3). No bioaccumulation factors (BAFs) in liver could be calculated.

3.2.2 Toxicity experiment

Turtle livers were analyzed for 37 individual or co-eluting PCB congeners and were presented as the sum of PCBs (Σ_{37}PCBs). For the toxicity experiment, the mean Σ_{37}PCB concentrations measured in the livers for each treatment were 0.47, 0.51, 0.66, 1.21, and 3.54 µg Σ_{37}PCB/g when exposed to pellets with 0, 0.09, 0.45, 2.3, and 12.7 µg Σ_{37}PCBs/g, respectively (Figure 3). The most abundant congeners in the liver at the highest treatment were, in order of abundance: PCB 118, 138, 153, 105, 99, 128, 170, 156, 158, 74, and 101 and they represented 87.5% (i.e., 3.01 µg Σ_{37}PCBs/g) of the total PCB concentration (Table S3 & Figure S5).

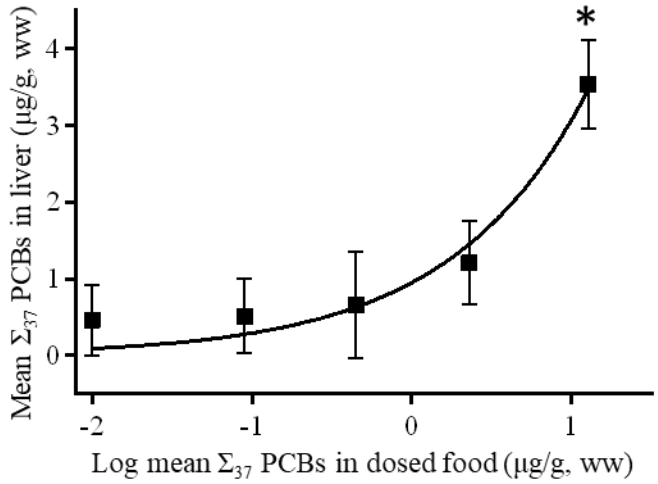

Figure 3. Σ_{37}PCBs/g in *C. s. serpentina* liver after an 81-day exposure to varying concentrations of A1254. Juvenile turtles were exposed to 0, 0.09, 0.45, 2.3, and 12.7 µg Σ_{37}PCBs/g. Data is presented as mean (n = 6-7) ± SD. Significance is denoted by an asterisk (*) for Σ_{37}PCBs. A linear trend line provided the best fit; $R^2 = 0.8066$.

BAFs were calculated for all congeners in livers from turtles by dividing the Σ_{37}PCBs/g in *C. s. serpentina* liver by the PCB concentration in the food of the highest treatment (12.7 µg/g). The

310 congeners measured had BAFs in liver up to 0.06 and a log K_{ow} (octanol/water partition
311 coefficient) ranging from 5.75 to 8.18 (log K_{ow} were based on Hawker & Connell, 1988).
312 Congeners with mid-range log K_{ow} (i.e., 6.5 – 7.0) exhibited the highest BAFs (Figure S4).
313 In addition, when all congeners were separated by metabolic groups, nine belonged to group 1
314 (non-metabolizable); six to group 2 (metabolized by CYP2B); 12 to group 3 (metabolized by
315 CYP1A); and 10 to group 4 (metabolized by both CYP1A and CYP2B) (Table 1). Of the 12
316 congeners that were significantly increased at the highest dose, the majority belonged to group 3
317 (PCB 170, 74, 99, 105, 118, 128, 138, 156, 158, and 171). The mean concentration of group 3
318 congeners was significantly increased (p = 0.0025) when exposed to 0.045, 2.7, and 12.7
319 Σ_{37}PCBs/g treatments compared to controls (Figure 4). No significant changes were observed for
320 groups 1, 2, and 4. The most abundant PCB congeners after 81 d were primarily penta- and hexa-
321 PCBs, in which the majority were ortho-meta unsubstituted congeners.
322 Table 1. PCB congeners measured and their corresponding metabolic group.

Metabolic group	**Measured congeners (IUPAC #)**
Group 1	153, 180, 183, 187, 194, 205, 206, 208, 209
Group 2	52, 95, 101, 149, 151, 199
Group 3	74, 99, 105, 118, 128, 138 ,156, 158, 170, 171, 177, 195
Group 4	18/17, 31/28, 33, 44, 49, 70, 87, 110

323

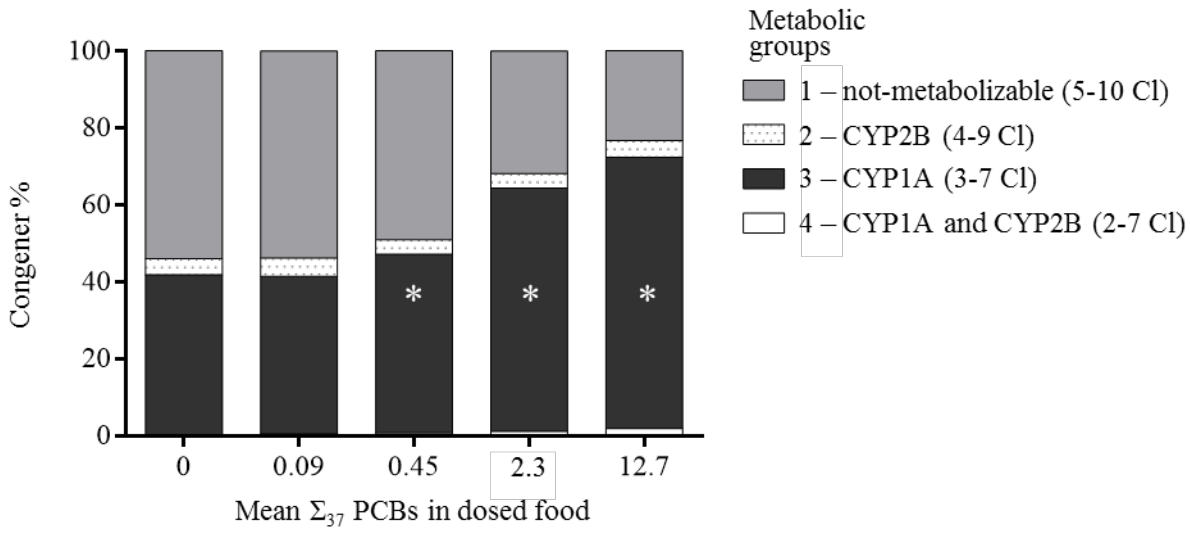

Figure 4. Percentage of congener metabolic groups measured in *C. s. serpentina* liver after an 81-d exposure to varying concentrations of A1254. Significant differences were observed in congeners in group 3 when exposed to 0.45, 2.3, and 12.7 µg Σ_{37}PCBs/g, represented by an asterisk (*) compared to controls (one-way ANOVA and Tukey test).

Furthermore, PCA was conducted on all congeners whose concentrations were above the LOD in liver for each A1254 treatment (i.e., PCB 101, 105, 118, 128, 138, 153, 170, 180, 183, 194, and 199). Components 1 and 2 explained 95% of the variance with 83.9% and 11.1%, respectively. As exposure concentrations of A1254 increased from 0 to 12.7 Σ_{37}PCBs, the congener profile in the turtle livers became increasingly more consistent with A1254 (Figure 5).

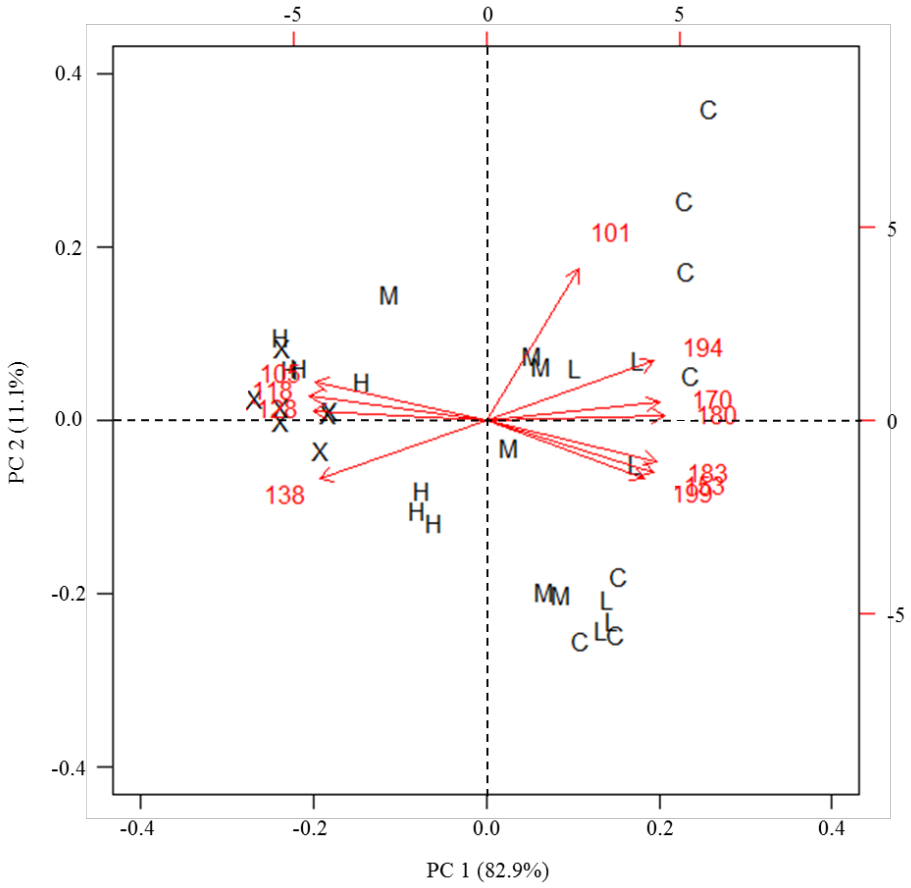

Figure 5. Principal component analysis (PCA) of PCB congeners in *C. s. serpentina* liver after an 81-day exposure to varying concentrations of A1254. Factor loadings of PCB congeners are presented by their respective IUPAC number. Factor scores of turtle liver samples are represented by their respective treatment (C = 0, L = 0.09, M = 0.45, H = 2.3, and X = 12.7 µg Σ_{37}PCBs/g). Note the PCA analysis was conducted on all congeners in liver that were above the limit of detection for each A1254 treatment (i.e., PCB 101, 105, 118, 128, 138, 153, 170, 180, 183, 194, and 199).

3.3 Gene expression

A series of 14 targeted genes involved in metabolism, oxidative stress, thyroid hormone axis, and reproduction were analyzed for expression in the turtle liver in the toxicity experiment. There was a significant 1.8-fold increase in *cyp1a* expression after exposure to 12.7 µg/g A1254 (p = 0.02, Figure 5) compared to controls. The *cyp1a* mRNA level increased as did the proportion of

PCB congeners belonging to the metabolic group 3 (metabolized by CYP1A). No other expression changes were observed in any other genes studied (Figures S6 & S7).

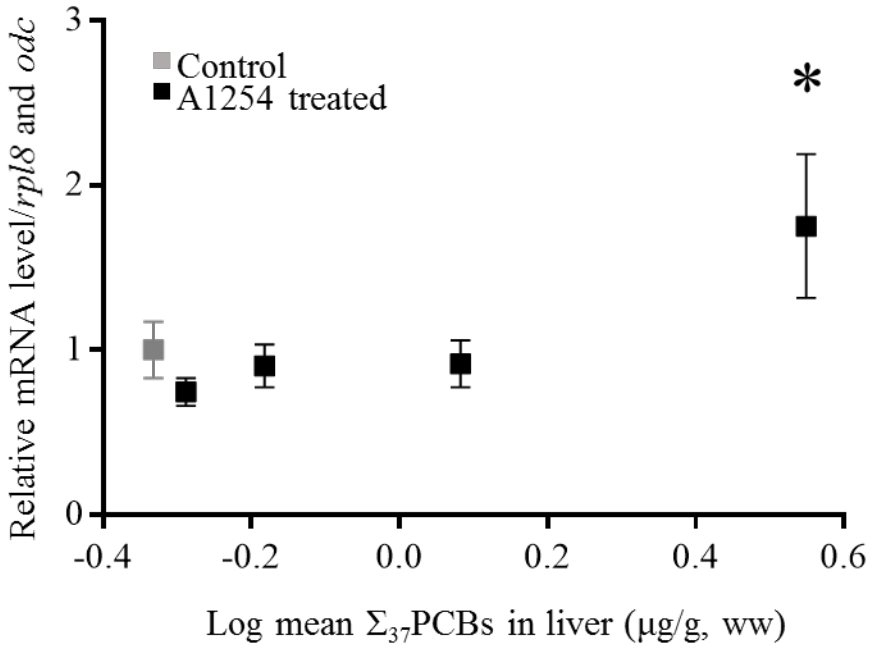

Figure 5. *cyp1a* gene expression in *C. s. serpentina* liver after exposure to 0 - 12.7 µg Σ_{37}PCBs/g. Data are shown as mean (n = 6-10) fold change + SEM. Significance ($p < 0.05$) is depicted by an asterisk (*) after a one-way ANOVA and Tukey test

4. Discussion

There has been a large amount of research on the toxicity of PCBs in vertebrates; however, very little is known as to how reptiles are affected by PCBs. This study aimed to assess the effects of a dietary exposure of PCBs to juvenile turtles in a controlled laboratory setting.

No changes were observed in PCB liver accumulation in the toxicokinetics study as the concentration of fed PCBs (0.45 µg Σ_{37}PCBs/g) was not elevated enough. This was evident when compared to the toxicity study, discussed below, in which statistically significant increase in liver PCB concentrations was not observed until the highest dose, 12.7 µg Σ_{37}PCBs/g, indicating that this concentration, although environmentally relevant, does not show significant hepatic

362 accumulation during acute exposure. A longer exposure, or a higher rate of feeding, may have
363 ultimately resulted in an increase in PCB burdens.
364 Liver PCB concentrations showed a dose-dependent linear accumulation in *C. s. serpentina* liver
365 after an 81-d exposure with increasing concentrations of A1254 in pellets, between 2.3 and 12.7
366 μg Σ_{37}PCBs/g. Low levels of PCBs found in the control food, and in the livers of turtle livers as
367 residual from maternal transfer from the females laying eggs, confounded relationships with
368 PCBs in the control and low dose groups. PCBs with low log K_{ow} values tend to be readily
369 absorbed and cleared due to their relatively smaller molecular size and relative ease of being
370 metabolized, while PCBs with high log K_{ow} values are not readily absorbed (Matthews et al.,
371 1978). The current study showed that congeners with mid-ranged log K_{ow} values (6.5-7)
372 generally yielded the highest BAF in liver.
373 PCA analysis showed an increase in mid-range chlorinated congeners in the liver at higher
374 treatments. As turtles are exposed to higher concentrations of A1254, the congeners that
375 increased the most in liver at higher treatments were also those which are most abundant in
376 A1254 (PCB 105, 118, 128, and 138) and have log K_{ow} associated with high BAFs (Schultz et
377 al., 1989). PCB congeners in eggs of wild *C. s. serpentina* similarly reflected the local Aroclor
378 sources of PCBs they were exposed to, for both A1254 and A1260 (de Solla et al., 2007). *C. s.*
379 *serpentina* eggs that were collected over a spatial gradient downstream of a point source of PCBs
380 in Lyons Creek (Welland, ON, CA) showed a similar pattern; eggs collected closer to the PCB
381 source (largely A1254), had PCB profiles that were increasingly similar to that Aroclor (de Solla
382 et al., 2007). These findings show that the profiles of PCBs in livers correspond to environmental
383 sources, and hence can be used in environmental forensic applications to determine point sources
384 of contamination.

385 Cytochrome P450 enzymes are responsible for xenobiotic metabolism, including PCBs, making
386 it an important biomarker of PCB exposure (van der Oost et al., 2003; Hofvander, 2006).
387 Consistent with Aroclor 1254, we found a significant increase in both concentrations and
388 proportions in the turtle livers of PCBs from congener group 3 (i.e., PCB congeners that can be
389 metabolized by CYP1A and have only *o*,*m* vicinal H-atoms), which both correlated to increases
390 in *cyp1a* mRNA levels. Similar results have been observed in other taxa; a 1.8-fold increase in
391 *cyp1a* expression was observed in whole *Danio rerio* (zebrafish) embryos exposed to water
392 spiked with 32 and 64 µg/L of the PCB 126 as early as 24 h post fertilization (hpf) until 168 hpf
393 (Liu et al., 2016). *Microgadus tomcod* (frostfish) showed a large *cyp1a* mRNA increase (77-fold)
394 seven days after an initial intraperitoneal injection of PCB 77 at 100 ppm (Yuan et al., 2006).
395 *Silurana tropicalis* (Western clawed frog) larvae showed a 77-fold increase in *cyp1a* expression
396 after being exposed to PCB 126 for 12 h in contaminated water (Jönsson et al., 2011). Similar
397 observations were noted in avian species; both *Gallus gallus* (chicken) and *Coturnix japonica*
398 *(Japanese quail)* embryo hepatocytes exhibited increases in *cyp1a4* mRNA levels (669 to 2,900
399 and 8.2 to 38.2-fold-change, respectively) and *cyp1a5* (115 to 254 and 5.7 to 12.2 fold-change,
400 respectively) after a 24 h exposure to either PCB 77, 105, 118, or 126 (Manning et al., 2013).
401 Given CYP1A's role in xenobiotic metabolism, the observed increase in *cyp1a* expression from
402 this study suggests that *C. s. serpentina* could metabolize PCBs through CYP induction. PCBs
403 that are metabolized by P450s form hydroxylated PCBs (e.g. Letcher et al. 2015), which i) are
404 more water soluble, and ii) more easily conjugated into even more water-soluble forms. With a
405 few exceptions, this increases the rate that the PCBs are e liminated, mostly in urine but also in
406 faeces. A few hydroxylated PCBs, such as HO-PCB187, will bind with transthyretin, a thyroid
407 hormone binding protein found in blood, and thus HO-PCB187 tends to reside in blood (e.g.

Letcher et al. 2015). Letcher et al. (2015) proposed that wild *C. s. serpentina* are capable of metabolizing PCBs into OH-PCBs, as their plasma concentrations of OH-PCBs were proportional with PCB concentrations. The current study in combination with Letcher et al. (2015) demonstrate that *C. s. serpentina* can likely metabolize PCBs, given the induction of enzymes responsible for the formation of OH-PCBs. Also, it is expected that the measured hepatic levels could have been significantly higher in the turtle's tissue if CYPs would not have been activated.

Xenobiotic metabolism can result in the formation of superoxide radicals and hydrogen peroxide due to metabolism from CYP1A, which can trigger reactive oxygen species (ROS) formation (Schlezinger et al., 2000). Given that PCBs increase oxidative stress in other aquatic species, we predicted that detoxification-related genes would be up-regulated to reduce ROS accumulation. However, no changes in detoxification-related gene expression were detected. Similar data were obtained in *S. tropicalis* tadpoles following a 12-h waterborne exposure to PCB 126; the expression of *gst* did not change, although expression of *sod1* increased (Jönsson et al., 2011). Exposure of up to 1.6 μg PCB 126/kg showed no changes in GPx activity in *Anas platyrhyunchos* (Jin et al., 2001). No changes in CAT, SOD, or GPx activity were observed after 30 weeks in *Salvelinus namaycush* (lake trout) after intraperitoneal injection of PCB 126, ranging from 0.6 – 24.8 ng/g (Palace et al., 1996). The lack of response of detoxification-related gene expression in turtles may in part be due to the turtle's ability to deal with anoxia, which is a stressor they are normally exposed to annually during hibernation. As turtles overwinter, they must survive anoxic to aerobic transitions, and therefore, maintain high antioxidant defence (Storey, 1996). Turtles maintain higher basal activity of enzymes required for reducing ROS accumulation such as CAT, SOD, and GST (Hermes-Lima and Zenteno-Savin, 2002). Given that

431 turtles have even slower metabolic rates than other non-fish vertebrates (reviewed in Nagy,
432 2005), it is expected that it would require a longer time-period to detect gene expression changes
433 related to detoxification in turtles.
434 There were no changes in thyroid hormone-related gene expression in turtle hatchings. In
435 contrast, dose-dependent increases of thyroid hormone-related genes were found in other species.
436 For example, *Paralichthys olivaceus* (olive flounder) showed an increased expression of *dio3* in
437 the liver after 25- and 50-day waterborne exposures of 10 to 1,000 ng/L A1254 (Dong et al.,
438 2014). In *Oreochromis niloticus* (Nile tilapia), liver DIO3 protein levels were significantly
439 higher following a diet of 0.5 μg/g A1254 for 21 and 35 days (Coimbra et al., 2005). The
440 increase in DIO3 activity suggests an abundance of triiodothyronine or thyroxine production
441 (Coimbra et al., 2005). Gauger et al. (2007) suggested that PCB 105 and 118, once metabolized
442 by CYP1A can act as thyroid hormone agonists, competing for binding to thyroid hormone
443 receptors. The discrepancy between these fish studies and the present one could be a difference
444 in species sensitivity.
445 In the current study, no changes in HPG-related gene expression were observed. Similar results
446 were observed in wild *Caretta caretta* (loggerhead sea turtle) where no changes were observed
447 for *esr1* in turtle's plasma contaminated with PCB 52, PCB 95, and PCB 149 (Cocci et al. 2018).
448 Yum et al. (2010) also demonstrated that expression of *esr1* remained unchanged after 24-h
449 exposure to 100 μg/L of A1260 in *D. rerio*. In contrast, Matsumoto et al. (2014) observed male
450 to female sex reversal in *Trachemys scripta elegans* (red-eared slider turtle) eggs after a one-time
451 injection of a mixture of 4'-OH-PCB 61 and 4'-OH-PCB 30, and 4'-OH-PCB 30 alone. In
452 addition, upregulation of ovarian markers (i.e., aromatase, forkhead box L2, and R-Spondin 1)
453 were noted in *Trachemys scripta elegans* exposed to both OH-PCBs (Matsumoto et al., 2014).

Measuring OH-PCB concentrations and comparing to sex steroid-related gene expression may show a correlation in *C. serpentina*, given OH-PCBs stronger affinity to ESRs, than PCBs. Both ecological and toxicological studies have found maternal effects, where there are alterations to the offspring's phenotype due to the mother's environment. For example, nest site choice by female turtles allows mothers to partially control the environment of the incubating eggs, affecting the growth and development of hatchlings (Mitchell et al., 2015). Further, previous maternal exposure to high levels of PCBs increased the toxicity of later dietary exposure to PCBs (Eisenriech et al., 2009). The eggs we collected had fairly low PCB burdens; it is possible that if we collected eggs from more contaminated sites, we may have seen a different response to dietary PCB exposure.

5. Conclusion

PCBs are currently ubiquitous in the environment and wildlife, and it is therefore crucial to understand the mechanisms behind PCB toxicity in long-lived vertebrates. As such, long-lived animals that have small home ranges may be a good indication of ecosystem health as their contaminant burdens would reflect the exposure from their local habitat. In this study, we demonstrated that *C. serpentina* could indeed reflect dietary sources of PCBs in its liver. Furthermore, our data suggested that turtles induce *cyp1a* mRNA levels with PCB dietary exposures equal or higher than 12.7 µg/g, ww, which corresponded to liver burdens of 3.54 µg Σ_{37}PCB/g, ww. In the lower Great Lakes region, concentrations of PCBs in snapping turtle eggs tend to range between 0.17 to 1.4 µg/g, ww, although concentrations exceeding 6 µg/g in turtle eggs are still found (Hughes et al., 2019). Hence, PCB concentrations in wild turtles sometimes exceed those we found in our study to induce CYP1A. PCB exposures resulting in body burdens similar to those found in wild turtles may induce CYP1A, and thus, putatively increasing PCB

elimination through increased formation of metabolites. Furthermore, investigating the role of OH-PCBs in the turtle's endocrine system would be an asset given these metabolites are known to affect HPT and HPG in other vertebrates.

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