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BIOACCUMULATION OF ORGANOCHLORINE CONTAMINANTS AND ETHOXYRESORUFIN-*O*-DEETHYLASE ACTIVITY IN SOUTHERN CALIFORNIA ROUND STINGRAYS (*UROBATIS HALLERI*) EXPOSED TO PLANAR AROMATIC COMPOUNDS

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Abstract: While contaminant concentrations have been reported for elasmobranchs around the world, no studies have examined bioaccumulation patterns across male and female age classes. The round stingray (*Urobatis halleri*) is a local benthic species that forages near areas of high organochlorine contamination and represents a good elasmobranch model. Polychlorinated biphenyls (PCBs), DDT, and chlordanes were measured in juvenile and adult male and female stingrays from areas in southern California, USA ($n = 208$), and a nearby offshore island, Santa Catalina ($n = 34$). Both mainland juvenile male and female stingrays showed a significant dilution effect. After maturity, summed contaminant concentrations significantly increased with size for adult males (median 11.1 μg/g lipid wt) and females (5.2 μg/g lipid wt). However, the rate of bioaccumulation was substantially greater in male stingrays than in females, likely a result of the females' ability to offload contaminants to offspring during pregnancy. In addition, males and females showed significant differences in their contaminant profiles, suggesting differential habitat use. Male and female stingrays collected from Santa Catalina Island had significantly lower concentrations (0.51 μg/g and 0.66 μg/g lipid wt, respectively), approximately 5 times less than those of mainland animals. Potential toxicity effects mediated through activation of the aryl hydrocarbon receptor were explored through ethoxyresorufin-*O*-deethylase (EROD) activity assays. Mainland male stingrays exhibited significantly greater EROD activities than Catalina males (481 pmol/min/mg protein and 55 pmol/min/mg protein, respectively); however, activity levels in female stingrays from both locations were comparable (297 pmol/min/mg protein and 234 pmol/min/mg protein, respectively) and lower than those in mainland males. The results suggest that PCBs and/or other structurally related contaminants may be inducing a biological response in mainland males but not females, possibly the result of a dampening effect of estradiol; however, the exact physiological repercussions of exposure remain to be determined. *Environ Toxicol Chem* 2014;33:1380–1390. © 2014 SETAC

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INTRODUCTION

Organic contaminants such as polychlorinated biphenyls (PCBs), chlordanes, and DDT and metabolites have been documented to accumulate to high levels in upper trophic–level marine mammal predators such as cetaceans [1], pinnipeds [2], and polar bears [3]. Recently, contaminant research in other marine predators such as elasmobranch fishes has increased knowledge of the degree to which these k-selected species also acquire high contaminant loads [4–6]. Similar to marine mammals, many elasmobranchs occupy relatively high trophic positions [7] and have a high lipid storage capacity via large livers, where contaminants accumulate and can concentrate to high levels [8,9].

In marine mammals, contaminant concentrations can vary greatly among individuals depending on factors such as age and sex [10,11]. Reproductive marine mammal females tend to have significantly lower levels than mature males [2,12] because of the females' ability to offload a majority of contaminant burden to offspring during pregnancy and lactation [13]. Males and juveniles, on the other hand, do not have corresponding pathways to rid their bodies of lipophilic contaminants [13]. Therefore, contaminant levels in males and subadults tend to increase with age until input of contaminants is balanced by loss through metabolism and excretion through the digestive or urinary system [13,14]. Many elasmobranch studies have also documented wide variability in contaminant concentrations measured among individuals [4,6]; however, very few have taken into account the contribution of gender and age to this variability, because little is known about how these factors influence organic contaminant bioaccumulation rates.

Measuring basic contaminant concentrations is necessary to understanding the extent of bioaccumulation in species; quantifying organisms' biological responses to exposure is critical, however, to determe if contaminants are having any negative, measurable effects. Enzymes of the cytochrome P450 family (CYP), which are found in a wide range of taxa, act as useful biomarkers to demonstrate the bioavailability of contaminant exposure [15]. In particular, the CYP1A enzyme, which is involved in the first phase of xenobiotic detoxification [16], is upregulated with exposure to coplanar aromatic hydrocarbons, such as some dioxin-like PCBs and polycyclic aromatic hydrocarbons (PAHs), through binding the aryl hydrocarbon receptor (AhR). Since its expression and activity can easily be measured, CYP1A is a useful biomarker of exposure that can be used to demonstrate biochemical responses to certain classes of organic contaminants [17,18]. Quantifying CYP1A activity through the ethoxyresorufin-*O*-deethylase (EROD) assay is a widely used technique to indirectly measure the amount of expressed enzyme [19], which measures the rate of resorufin production (a fluorescent compound). Furthermore,

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induction of the CYP1A system has been correlated to negative effects in vertebrates such as DNA adducts [20], cancer [21], and reproductive disorders [22].

Biochemical responses to contaminant exposure by induction of the CYP1A system have been previously documented to occur in elasmobranch species ([23–25]; C.J. Walker, 2011, Master's thesis, University of North Florida, Jacksonville, FL, USA). However, none of these studies examined the influence of factors such as sex, season, or diet, which have been shown to affect the activity or expression of this enzyme [26]. For example, estrogens may significantly reduce the expression of CYP1A in mature females [27]. Therefore, factors known to influence CYP1A, such as sex, should be considered when interpreting results, to ensure that appropriate conclusions are made about the response of elasmobranchs to contaminant exposure.

The high mobility of elasmobranchs, invasiveness of tissue acquisition (e.g., liver tissue sampling), and difficulty in obtaining samples of specific age classes have limited contaminant research in these species as well as our understanding of bioaccumulation trends and effects on physiology. The round stingray (*Urobatis halleri*) is an abundant benthic forager found coastally throughout southern California, USA [28]. The stingray's close proximity to highly contaminated urban areas [29] and accessibility to a wide range of size classes makes this species an appropriate elasmobranch model to examine contaminant bioaccumulation and bioavailability. In addition, a genetically distinct population of round stingrays is present at a nearby offshore island, Santa Catalina [30], located approximately 35 km away from the mainland. The deep channel separating the island from the mainland has greatly limited contamination and gene flow, making Santa Catalina Island a relatively less contaminated area compared with the mainland and presenting a good natural reference site for comparison; previous studies have found a range of taxa having significantly lower contaminant concentrations [31,32]. Therefore, the goals of the present study were to determine and compare bioaccumulation patterns among different age classes and between male and female stingrays, to quantify hepatic EROD activity as an indicator of biochemical response to contaminant exposure, and to compare contaminant accumulation patterns and responses of stingrays sampled from highly and less polluted areas in southern California, notably Santa Catalina Island.

MATERIALS AND METHODS

Sample collection

Mainland stingrays (*U. halleri*) were collected in summer (June–July) and fall (September) 2011. Males from several age classes and juvenile females were collected primarily from Seal Beach, California, USA, and mature females were collected from Colorado Lagoon and the Seal Beach National Wildlife Refuge, California, USA (Supplemental Data, Figure S1). To limit the number of animals that were sacrificed, we operated under the assumption that prior to maturity males and females would bioaccumulate contaminants similarly. Because contaminant accumulation in males is not confounded by reproduction as it is in females [10,12], male stingrays were subdivided into 4 age class groups using previously published age/growth curves [33] (Table 1) to allow us to examine in detail bioaccumulation with age and particularly around the point of maturity

Table 1. Size class ranges and the median and range (minimum–maximum, outliers) for organochlorine concentrations (μg/g lipid wt) and EROD activities (pmol/min/mg protein) are presented for sampled stingrays[a]

Location	Sample group	Size range	Summed OCs	PCBs	CHLs	DDTs	EROD activity
Mainland							
	Males						
	Juveniles 1	11–12.8	5.6 (24)	4.7	0.31	0.74	925 (19)
			3.1–17.1	2.5–16.1	0.14–0.55	0.39–1.3	189–1408
	Juveniles 2	13.0–14.0	4.8 (27)	3.7	0.22	0.45	688 (23)
			2.1–16.5	1.4–13.5	0.07–0.81	0.11–2.4	104–1207
	Juveniles 3	14.5–16.5	3.4 (32)	2.8	0.15	0.34	705 (27)
			1.9–9.0	1.5–8.3	0.067–0.38	0.16–0.62	219–1067
	Adults	16.8–22.4	11.1 (28)	9.5	0.57	0.59	481 (22)
			3.2–31.3, 50.4[b]	2.6–31.1, 48.2[b]	0.085–2.8	0.085–2.3	177–1181
	Females						
	Juveniles	11.8, 14–16.5	2.7 (12)	2.2	0.13	0.40	297 (12)
			1.4–9.4	1.1–7.7	0.015–0.46	0.18–1.2	114–492, 1306[b]
	Adults	15.7–33.3	5.2 (85)	4.2	0.23	0.61	272 (75)
			1.9–20.6	1.5–17.0	0.055–2.4	0.20–2.4	60–988, 1408[b]
Santa Catalina Island							
	Males						
	Juvenile	16.1	1.2 (1)	1.1	0.02	0.13	328 (1)
			—	—	—	—	—
	Adults	18.7–25.8	0.51 (10)	0.47	0.025	0.017	55 (8)
			0.27–1.6, 3.4[b]	0.23–1.4, 3.1[b]	0.008–0.075	0.008–0.28	44–73, 272[b]
	Females						
	Juveniles	13.9–16.9	0.79 (3)	0.63	0.04	0.10	326 (3)
			0.068–1.2	0.05–1.1	0.024–0.058	0.054–0.15	285–886
	Adults	17.5–26.9	0.66 (20)	0.57	0.033	0.076	234 (19)
			0.31–1.6, 4.5[b]	0.22–1.6, 4.3[b]	0.008–0.06, 0.3[b]	0.004–0.19	60–448

[a]Stingray size class ranges are presented as disk width (cm). Sample sizes for organochlorine and EROD analysis are shown in parentheses. Sample size discrepancies between organochlorine contaminants and EROD activities were the result of an inability to properly store (i.e. at −80 °C) some samples at the time of sampling.
[b]Extreme outliers.
OC = organochlorine; PCB = polychlorinated biphenyl; CHL = chlordane compound; DDT = dichlorodiphenyltrichloroethane compounds; EROD = ethoxyresorufin-O-deethylase.

(approximately 15 cm disk width) when males and females may begin to exhibit reproductive differences.

Animals were collected within ± 2 h of the low tide using a large or a small beach seine net at Seal Beach and nearby estuaries, respectively. Upon capture, stingrays were sexed and disk widths measured. A subset of stingrays was transported back to the Shark Lab at California State University, Long Beach, (CSULB) where stingrays were euthanized by immersion in a seawater ice slurry for 30 min followed by spinal pithing, in agreement with approved CSULB Animal Welfare Board Protocol 273. Once rays were euthanized, disk width, total body, gonad weight, liver weight, and clasper length (males) were obtained. Two pieces of the left liver lobe were sampled and immediately frozen at −20 °C and −80 °C, respectively. The remaining liver was stored at −20 °C.

Stingrays were collected from Santa Catalina Island during summer and fall (August–October) 2011 and 2012 (Supplemental Data, Figure S1, inset B). Mature males were primarily sampled from Empire Landing by dipnet using SCUBA ($n = 7$). Females ($n = 23$) and remaining males ($n = 4$) were collected by small beach seine or hook and line from Catalina Harbor. Stingrays were measured, and samples of liver were obtained and frozen after rays were euthanized by the methods described above.

Organic contaminant quantification

Organochlorine contaminant quantification was performed at the CSULB Institute for Integrated Research on Materials, Environment and Society. Each sample extract was analyzed for 3 groups of contaminants: PCBs (54 congeners), chlordane compounds (oxychlordane, *trans*- and *cis*-chlordane, *trans*-nonachlor), and dichlorodiphenyltrichloroethane (DDT) and its metabolites (2,4′-dichlorodiphenyldichloroethylene [DDE], 4,4′-DDE, 2,4′- dichlorodiphenyldichlorethane [DDD], 4,4′-DDD, 2,4′-DDT, 4,4′-DDT; collectively, "DDTs"). Compounds measured were summed to obtain total PCBs, chlordanes, and DDTs for their respective groups as well as pooled among groups to obtain total concentrations of organochlorines for each stingray sample. In addition, total concentrations of a subgroup of PCBs (coplanar) known to induce the CYP1A system (PCBs 81, 123, 114, 105, 126, 167, 156, 157, 169, 189) were summed to obtain concentrations of CYP1A inducers (hereafter, "coplanar PCBs").

Following the methods of Lyons et al. [34], subsamples of livers frozen at −20 °C were extracted for 14 h to 16 h via a Soxhlet apparatus in 100% v/v methylene chloride solution. Prior to extraction, all samples were spiked with a known quantity of recovery surrogates (tetrachloro-m-xylene, PCBs 30, 112, and 198) to measure the efficiency of preparative and analytical procedures (target recovery of 70–130%). After extraction, samples were concentrated by rotovap, and lipid content was determined gravimetrically from split aliquots. Extracts were then purified through elution with an Alumina-B/Silica gel with hexane (15 mL), 30% v/v methylene chloride in n-hexane (30 mL), and methylene chloride (15 mL) and concentrated. All samples were spiked with internal standards (4,4'-dibromobiphenyl and 2,2',5,5'-tetrabromobiphenyl) and injected onto an Agilent gas chromatograph (GC; 6890N series) equipped with a mass selective detector (Agilent 5973 inert series). The GC column employed was a ZB-5 (Phenomenex) fused silica capillary (0.25 mm inner diameter × 60 m) with 0.25-μm film thickness. The temperature profile of the GC oven was programmed from 45 °C to 125 °C at 20 °C/min, then to 295 °C at 2.5 °C/min, and held for 10 min. Injector and transfer line temperatures were set at 285 °C and 300 °C, respectively. The source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Helium was used as the carrier gas at a flow velocity of 40 cm/s. The mass selective detector was operated in electron ionization mode and scanned from 45 amu to 500 amu at a rate of 1.66 scans/s. Concentrations of organic contaminants were quantified using the software in the GC/mass spectrometry system (Agilent Technologies) and reported on a lipid weight or wet weight basis.

Quality-assurance and quality-control samples were run in tandem with each batch ($n = 17$) of study samples to ensure the accuracy and precision of the data acquired and included 1 blank, 1 study sample replicate, 2 duplicate matrix spikes, and 1 certified reference material (Lake Michigan trout tissue 1947, National Institute of Standards and Technology). Matrix spikes were prepared by adding spike surrogates to subsamples used for pesticide and PCB analysis. The quality-control goal was for 90% of the replicates to yield a relative percent difference of <30% with recovery of spiked analytes at 70% to 130%.

The mean percentages (± standard deviation [SD]) of recovery of surrogates were 117 ± 25%, 115 ± 74%, 130 ± 23%, and 97 ± 20% for tetrachloro-m-xylene, PCB 30, PCB 112, and PCB 198, respectively, which demonstrated acceptable efficiency of procedures. Blanks showed no signs of external contamination during procedures. Certified reference material analytes were recovered within acceptable ranges (95 ± 3% for PCBs and 93 ± 6% for pesticides), and the mean ± SD relative significant differences between replicates of duplicate and matrix spike samples were 15 ± 15% and 7 ± 8%, respectively. Average recovery of matrix spikes was 92 ± 4% for PCBs and 85 ± 9% for pesticides. Therefore, samples met quality-assurance and quality-control criteria.

CYP1A quantification

Microsomal fractions were prepared following the methods of Lavado et al. [35] by homogenizing livers (~1 g) in a 1:5 w/v of cold 100 mM $KH_2PO_4/KHPO_4$ buffer (pH 7.4) with 150 mM KCI and 1 mM ethylene-diaminetetraacetic acid (EDTA). Homogenates were then centrifuged at 12 000 *g* for 30 min (4 °C) to remove the fatty layer, and the supernatant was centrifuged a second time at 100 000 *g* for 1 h (4 °C). The microsomal pellets were resuspended in a small volume of 100 mM $KH_2PO_4/KHPO_4$ buffer (pH 7.4) with 150 mM KCI, 1 mM EDTA, and 20% w/v glycerol and separated into 3 aliquots. Protein content was determined using the Bradford method with bovine serum albumin (BSA) as the standard [36].

Cytochrome P450 was quantified using a competitive enzyme-linked immunosorbent assay (ELISA; Biosense) with mouse anti-fish CYP1A monoclonal antibody (Biosense) as the primary antibody. Cross-reactivity of the primary antibody with stingray CYP1A protein was verified through Western blot in a 1:1000 dilution (v/v) using a rainbow trout (*Oncorhynchus mykiss*) that had been injected with benzo[*a*]pyrene as a positive control (Supplemental Data, Figure S2). The ELISAs were carried out according to the manufacturer's instructions using reagents provided in the kit. Briefly, 100 μL of coating buffer (50 mM carbonate-bicarbonate, pH 9.6) was added to each well of a 96-multiwell plate and incubated at 4 °C overnight with 100 μL of stingray liver microsomes, which had been diluted to 40 μg/mL with coating buffer. Wells were washed 3 times with washing buffer (phosphate buffered saline [PBS], 0.05% w/v Tween-20) and incubated with 100 μL of diluted primary antibody (1:1000 v/v in 1% BSA–PBS solution) at 37 °C for 1.5 h. After incubation, wells were washed 3 times, and 100 μL

of the secondary antibody (horseradish peroxidase conjugate, diluted to 1:2000 v/v in blocking buffer 1% BSA–PBS) was added to wells and incubated at room temperature for 1.5 h. Wells were washed 5 times, and plates were developed by adding 100 μL of substrate solution (*o*-phenylenediamine peroxidase substrate) and incubating at room temperature for 25 min. The reaction was stopped by adding 50 μL of 2 M H_2SO_4 (Fisher Scientific). Absorbance was measured at 492 nm using a Varioskan Flash spectral scanning multimode reader (Thermo Scientific). Samples were run in duplicate ($4 \pm 4\%$ average relative SD), and each plate included a blank (nonspecific binding well) and a "positive control" stingray sample (JM2-20). Absorbances were blank-corrected and standardized to the absorbance of JM2-20, and sample duplicates were averaged. Therefore, stingray CYP1A contents represent a relative value.

The EROD activity was quantified in liver microsomes at room temperature following the procedures of Lavado et al. [37]. Briefly, microsomes (100 μg protein) were brought up to a volume of 500 μL with a 100 mM $KH_2PO_4/KHPO_4$ buffer (pH 7.6) containing 1 mM $MgCl_2$, 1 μM NADPH, and 1 μM 7-ethoxyresorufin and incubated for 10 min or 20 min for males and females, respectively. Optimum protein content, reaction time, and substrate concentrations were determined empirically. Reactions were stopped with 100 μL of cold methanol and centrifuged for 5 min at 900 *g* for 5 min. Fluorescence was measured using a Victor X2 Multilabel Plate Reader spectrofluorometer (PerkinElmer) at 537 nm excitation and 583 nm emission wavelengths and compared against a resorufin standard curve (10 nM–0 μM) run in triplicate. Each sample was run in pairs with duplicates ($n = 4$/sample) and a blank with each pair. Blanks consisted of all reaction materials, including the stingray microsome, except reduced nicotinamide adenine dinucleotide phosphate (NADPH) was not added.

Southern California sediment contaminants

Concentrations of PCBs (43 congeners), DDT and metabolites (2,4′-DDD, 2,4′-DDE, 2,4′-DDT, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT; $n = 6$), α- and γ-chlordane, and PAHs (22 compounds) for sediment samples ($n = 384$) from throughout Southern California were obtained from the Southern California Coastal Water Research Project Agency's Bight 2008 survey [38]. Contaminant concentrations from each sediment sample were summed to obtain total concentrations for each of the 4 respective contaminant groups and visually displayed using ArcMap 10 software (ESRI, 2003).

Statistical analyses

Bioaccumulation between males and females. The natural logarithms of lipid-normalized organochlorine concentrations (summed organochlorines, PCBs, chlordanes, and DDTs) were compared against disk width within gender groups for adults and juveniles using linear regression to examine accumulation rates within each size class by sex ($\alpha = 0.05$ for all tests). In addition, pairwise comparative tests were used to determine if the natural log of contaminant concentrations differed between size classes within male and female groups. In males, contaminant concentrations were compared among the 4 size classes (3 juvenile categories and 1 adult) using Kruskal-Wallis rank sum tests, whereas *t* tests were used for females because there were fewer juvenile samples (Table 1). In addition, PCB profiles were constructed for each of the 4 male size classes by dividing the quantified congeners into groups based on the number of chlorine atoms present and then calculating the percentage that each chlorinated congener group contributed to the total PCB load. The percentage of each PCB congener was compared among the 4 male age classes using a generalized linear model with a beta distribution and a logit linked function (SAS 9.3; SAS Institute). Within male and female groups, EROD activity was compared among size classes using Kruskal-Wallis tests or Welch's *t* tests. Linear regression was also used to explore the relationship between EROD activity with respect to the natural log sum of coplanar PCBs (wet weight) and disk width within male and female groups. All statistical tests were performed using the R statistical package (R Development Core Team).

Bioaccumulation between males and females. An analysis of covariance (ANCOVA) was used to determine if the rate of contaminant accumulation (i.e., natural log summed organochlorines, lipid weight) was different between males and females with increasing disk width. Because of the low fit of the linear regression, the relationship of natural log summed organochlorines with respect to disk width was compared between males and females using nonlinear regression and a second-order smoothing curve (32 neighbors), respectively. In addition, we were interested in determining if actual contaminant concentrations differed between males and females at various size and age classes. Therefore, pairwise comparisons of natural log PCB, chlordane, and DDT contaminant concentrations (lipid wt) were performed between males and females for adult and juvenile size classes using Welch's *t* tests. Previously published age/growth curves for male and female round stingrays were used to estimate ages of sampled males and females [33]. Natural log summed organochlorines were then compared using Welch's *t* test between comparably aged adult males and females as a second method to verify if bioaccumulation differs between the sexes.

Relative contaminant profiles were also compared between males and females by 2 methods. Percentages of PCBs, chlordanes, and DDTs were calculated by dividing each contaminant group by summed organochlorine concentrations per sample. The relative contributions of each contaminant group were then compared between males and females across disk width using 2nd-order smoothing curves. The second method involved comparing contaminant profiles between males and females by age class (i.e., adults and juveniles). Each measured contaminant was normalized against the most abundant and recalcitrant chemical (i.e., PCB 153) per sample [39]. Differences in organic contaminant profiles among species were analyzed using an analysis of similarity (ANOSIM), followed by a similarity of percentages (SIMPER) analysis using the Primer-6 software package [40]. Profiles were depicted by multidimensional scaling plots using Bray-Curtis similarity matrices. Differences in EROD activity and the natural log sum of coplanar PCBs between males and females for adult and juvenile age classes were analyzed using Welch's *t* tests.

Mainland versus Santa Catalina Island. Bioaccumulation patterns were determined in rays from Santa Catalina Island by regressing natural log summed organochlorines (lipid wt) against disk width by sex. Concentrations of natural log summed organochlorines of stingrays from the mainland and Catalina Island were compared by sex using Wilcox rank sum tests and Welch's *t* tests for males and females, respectively. Because of the lack of juvenile Catalina samples, we were unable to compare concentrations by both sex and age class. However, relative proportions of each of the 3 contaminant groups (i.e., PCBs, DDTs, chlordanes) and EROD activities were compared between the sexes for rays sampled at the mainland and Santa Catalina Island using Welch's *t* tests after percentages were

arcsin-transformed. Because of the large difference in sample size between the 2 sites, we compared relative CYP1A content from Catalina Island male stingrays to a randomly generated data set using a Monte Carlo simulation with the original mainland male data. The generated data set was created by randomly selecting 6 mainland stingrays with replacement (out of a possible 84 samples) 1000 times (R Core Team). Each randomly selected group of 6 mainland males ($n = 1000$) was then compared with the Catalina Island male group ($n = 6$) using a Welch's *t* test. By randomly selecting a sample size of mainland male stingrays that was comparable to our existing Catalina Island sample size, we can determine the frequency that any 6 randomly selected mainland males would have a statistically similar CYP1A content to that measured in Catalina Island males. Catalina females where CYP1A ($n = 3$) was measured were too low in sample size to statistically compare. The percentage of lipid content was compared between mainland and Catalina males and juvenile females from both locations. Mature females were unable to be compared because of differences in gestational stage (i.e., embryos were at different developmental points).

RESULTS

Bioaccumulation within male and female groups

Although summed organochlorines generally increased with size ($F_{1,110} = 38$, $p < 0.001$, $R^2 = 0.25$), the trend among males was better described by a quadratic relationship ($R^2 = 0.38$; Figure 1), whose inflexion point occurred at an approximate disk width of 15 cm. In addition, males exhibited differential bioaccumulation rates when individual contaminant groups (i.e., PCBs, chlordanes, DDTs) were separated according to adult and juvenile size classes. Juveniles showed a very distinct and significant decrease for all contaminant groups (i.e., summed organochlorines, PCBs, chlordanes, DDTs) with disk width ($F_{1,81} = 25.1$–40.5, $p < 0.001$, $R^2 = 0.22$–0.33). Adult males, on the other hand, only showed positive increases with size for summed organochlorines and PCBs ($F_{1,27} = 4.5$, $p = 0.043$–0.047, $R^2 = 0.11$). When contaminant concentrations were compared among age classes, adult males had significantly greater concentrations of PCBs and chlordanes than all 3 juvenile size classes ($W_3 = 56$–57, $p < 0.0001$; Table 1). However, adult DDT concentration was not significantly different from the 2 youngest male size classes ($p = 1.0$ and 0.88, respectively). Liver weight showed a significant positive linear relationship with disk width in males ($F_{1,110} = 486$, $p < 0.0001$, $R^2 = 0.81$; Figure 1); however, hepatic lipid content showed a negative quadratic relationship with increases in male disk width.

The PCB profiles were similar among male size classes ($F_3 = 0.85$, $p = 0.4$), with hexachlorinated congeners comprising a majority of total PCB concentrations (54%–59%; Figure 2). While the PCB proportions were significantly different among congener groups ($F_6 = 850$, $p < 0.001$), there was a significant interaction between age class and PCB congener group ($p < 0.001$). Juvenile stingrays had substantially larger proportions of the lower chlorinated congener groups (tri, tetra, and penta groups) that were approximately twice those measured in adults, which had greater proportions of the higher chlorinated groups (hexa to deca congeners). However, the smallest size class of males had similar proportions of deca congeners (2.2% and 1.9%, respectively), which was greater than the other 2 juvenile age classes (~1.1%). Median concentrations of each PCB congener group for the 4 male age classes can be found in the Supplemental Data, Table S1.

While there was no relationship between EROD activity and disk width ($F_{1,89} = 0.39$, $p = 0.53$), EROD activity was significantly different among size classes of males ($W_3 = 9.0$, $p = 0.03$). Adult males had significantly lower EROD activities only when compared with the youngest male size class ($p = 0.036$); however, all size classes were highly variable (Table 1). Although there was a significant increase in coplanar PCB concentration with disk width ($F_{1,110} = 34.2$, $p < 0.001$, $R^2 = 0.23$), EROD activity did not have a detectable relationship to coplanar PCB concentrations ($F_{1,89} = 0.67$, $p = 0.41$).

Adult females had significantly higher concentrations for all contaminant groups than juveniles ($t_{95} = -2.8$ to 2.1, $p = 0.006$–0.014; Table 1). Unlike males, juvenile females did not demonstrate a significant decreasing relationship for any of the contaminant groups ($F_{1,10} = 2.7$–4.9, $p = 0.051$–0.13), although PCBs and chlordanes were just above the 0.05 significance level. Although females showed positive increases in all contaminant groups with disk width (data not shown), the fits of regressions were poor and about one-half of that for males for summed organochlorines (0.13 vs 0.25, respectively). Females also showed a very strong linear relationship between

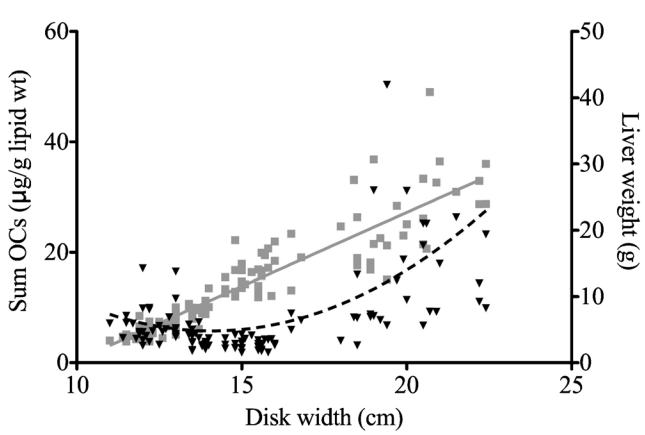

Figure 1. The natural log of summed organochlorine (OC) concentrations (lipid wt; black triangles) in male stingrays demonstrated a quadratic relationship with disk width (black dashed line; $R^2 = 0.38$). However, the relationship of liver weight to disk width (gray squares) was linear (solid gray line; $R^2 = 0.81$).

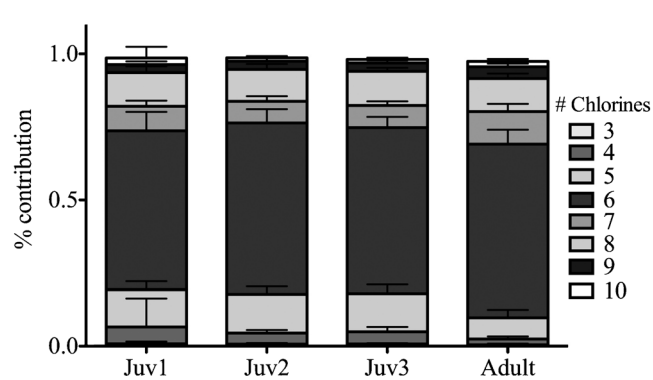

Figure 2. Mean (± standard deviation) proportions of chlorinated congener groups (tri–deca) varied between varying size classes of males. Adult males had higher contributions from the higher chlorinated groups (hexa–deca) compared with juvenile males (Juv), which had greater proportions of the lower chlorinated congeners (tri–penta).

liver weight and disk width ($F_{1,95} = 269$, $p < 0.0001$, $R^2 = 0.74$) and no relationship between hepatic lipid content and size ($F_{1,95} = 1.6$, $p = 0.2$).

Unlike that in males, EROD activity in females showed a significant decreasing relationship with disk width ($F_{1,85} = 9.3$, $p < 0.003$, $R^2 = 0.10$; Table 1) despite concurrent significant increases in coplanar PCB concentrations ($F_{1,85} = 31.1$, $p < 0.001$, $R^2 = 0.23$; Figure 3). These 2 regression lines intersected around the point when females were approximately 16 cm in size.When compared by age classes, however, there was no difference in EROD activity between juvenile and adult females ($t_{12.5} = 0.63$, $p = 0.54$). No relationship was observed between EROD activity and concentration of coplanar PCBs ($F_{1,85} = 0.8$, $p = 0.36$).

Bioaccumulation between males and females

When bioaccumulation patterns of summed organochlorines were compared between the sexes, males accumulated contaminants at faster rates compared with females ($F_{1,205} = 4.76$, $p = 0.03$; Figure 4A). In particular, the largest disparity occurred between larger sized males and females. Adult males had significantly higher concentrations of all contaminant groups (summed organochlorines, PCBs, and chlordanes; $t_{39} = -7.3$ to 6.9, $p < 0.001$), except for DDTs ($t_{39} = -0.41$, $p = 0.68$), compared with adult females. Furthermore, when similarly aged males and females were compared, males had significantly higher natural log summed organochlorine concentrations than females ($t_{42} = -7.8$, $p < 0.0001$; Figure 4B). Juveniles, on the other hand, showed no significant difference between males and females for any contaminant group comparison ($t_{16} = -1.8$ to 0.4, $p = 0.08$–0.66). Furthermore, males and females showed significantly different rates of liver growth with disk width (ANCOVA, $F_{1,200} = 14.6$, $p = 0.0001$).

In addition to different concentrations, contaminant profiles varied between males and females. With increasing disk width, males showed increases in the proportion of PCBs and simultaneous decreases in DDTs (Figure 5A). Whereas the proportion of chlordanes in males was fairly constant, females exhibited a substantial increase in chlordane proportion at approximately 19 cm disk width. The PCB proportions decreased in females with increasing size, but DDT remained relatively stable. Relative contaminant ratios also reflected these differences, with all sex and age classes being significantly different from one another (global $r = 0.309$, $p = 0.001$; Figure 5B). While no individual contaminant accounted for a large proportion of the differences among stingray age classes, 4,4′-DDE consistently was the most prominent contaminant responsible for the greatest separation among groups (approximately $10.22 \pm 0.65\%$), followed by PCB 138, PCB 118, and chlordanes.

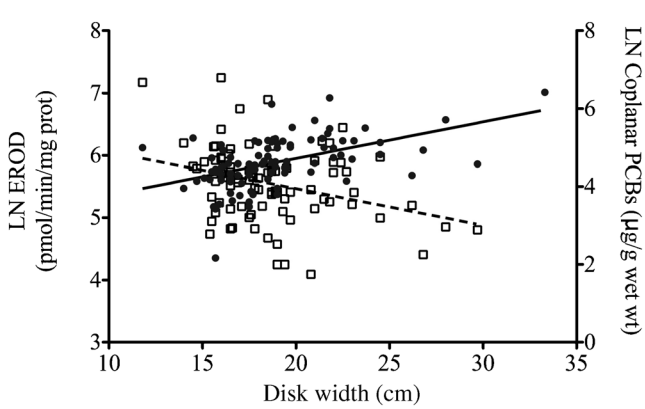

Figure 3. Levels of ethoxyresorufin-*O*-deethylase (EROD) activity (open squares) significantly decreased with disk width in mainland female stingrays (dashed line; $p < 0.003$, $R^2 = 0.10$), although coplanar polychlorinated biphenyl (PCB) concentrations significantly increased (black circles and solid line; $p < 0.001$, $R^2 = 0.23$). LN = natural logarithm.

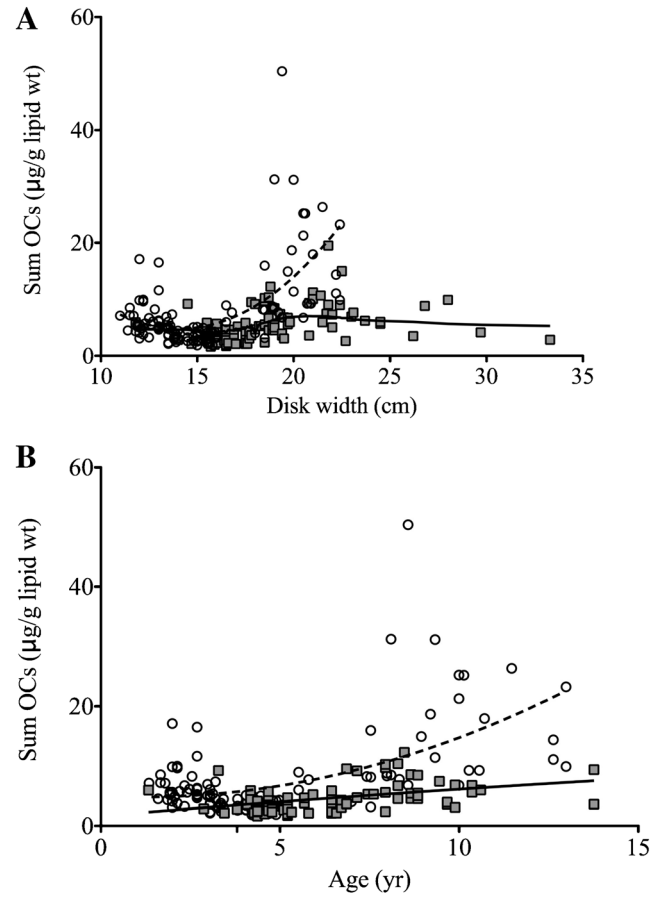

Figure 4. Male (open circles, dashed line) and female (gray squares, solid line) stingrays sampled from the mainland sites showed different patterns of contaminant bioaccumulation when compared by size (**A**) and age (**B**). Contaminant concentrations decreased with size in younger individuals and significantly increased in adults (around 15 cm disk width). Adult males had significantly greater contaminant concentrations compared with adult females when both age and size were considered. OC = organochlorine.

Levels of EROD activity were significantly greater in males than in females ($t_{16} = -5.9$, $p < 0.001$). Mature male activity was nearly twice that of mature females; however, mature females also had significantly lower concentrations of coplanar PCBs ($t_{40} = -2.5$, $p = 0.017$). Concurrently, relative CYP1A content was twice as low in females than in males ($t_{172} = -13.5$, $p < 0.0001$; Figure 6B). Juvenile females also had significantly lower EROD activities than juvenile males ($t_{15} = -3.1$, $p = 0.007$) despite having similar concentrations of coplanar PCBs ($t_{11} = -0.61$, $p = 0.55$). In addition, juvenile females had significantly lower relative CYP1A content, about half of that measured in juvenile males (W = 2500, $p < 0.001$).

Mainland versus Santa Catalina Island

Unlike mainland stingrays, males sampled from Santa Catalina Island showed a significant decreasing linear relationship between natural log summed organochlorines and disk

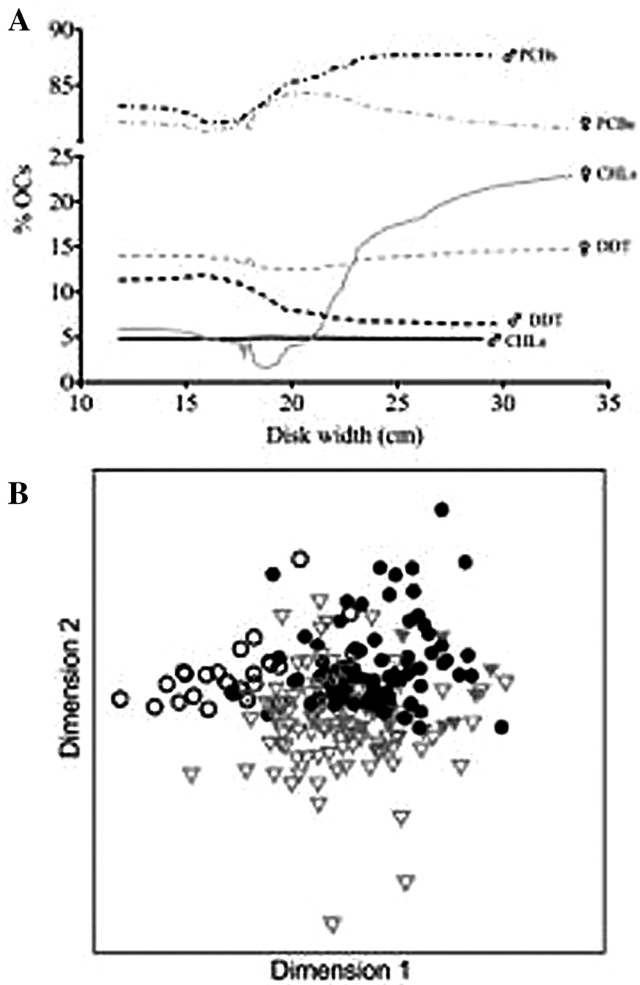

Figure 5. Mainland male and female stingrays exhibited differential contaminant profiles when proportions of (**A**) polychlorinated biphenyls (PCBs), DDTs, and chlordanes (CHLs), and (**B**) contaminant ratios were examined. (**A**) Trend lines for males are depicted in black and those for females in gray for PCBs (short dash), DDTs (long dash), and chlordanes (solid). Females exhibited a drastic increase in chlordane proportion with increases in size. (**B**) Multidimensional scaling plot: Stingrays were found to have significantly different contaminant ratios between males (black circles) and females (gray triangles) as well as between juvenile (closed) and adult (open) age classes (2D stress = 0.18). OC = organochlorine.

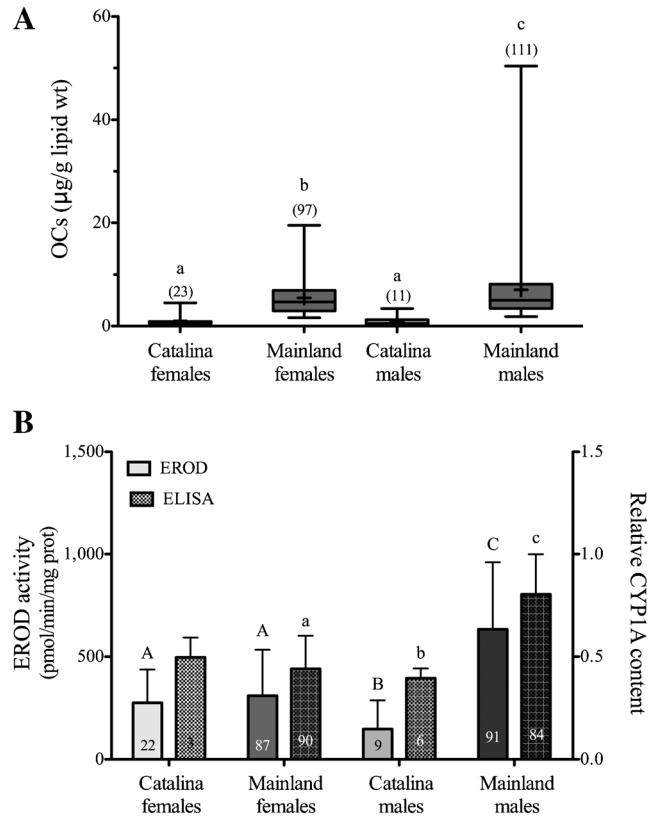

Figure 6. (**A**) Boxplots showing that contaminant concentrations measured in male and female stingrays from Santa Catalina Island (CA, USA; light gray shades) were significantly lower than those from their mainland counterparts (dark gray shades). Whiskers and crosses denote minimum and maximum values and group means, respectively. (**B**) Mean ± standard deviation ethoxyresorufin-*O*-deethylase (EROD) activity (open bars) and relative CYP1A content (hatched bars) did not show the same pattern. Females from both locations were not significantly different, whereas mainland males were significantly greater in their EROD activities and relative CYP1A content than Catalina males. For both graphs, different letters denote significant differences among groups, and sample sizes are placed above or within bars. Upper- and lowercase letters in (**B**) were independent group comparisons. OC = organochlorine; ELISA = enzyme-linked immunosorbent assay.

width ($F_{1,9} = 6.5$, $p = 0.03$, $R^2 = 0.36$), despite the fact that a large proportion of rays from the offshore island were greater in size than those sampled at mainland sites (Table 1). Females, on the other hand, showed no relationship with contaminant concentration and disk width ($F_{1,9} = 0.25$, $p = 0.62$). When Catalina rays were compared by sex, there was no significant difference in mean (± SD) natural log summed organochlorines between males (0.93 ± 0.93 µg/g lipid wt) and females (0.99 ± 1.0 µg/g lipid wt; $t_{20} = 0.19$, $p = 0.84$). However, females had significantly higher concentrations of coplanar PCBs than males (0.3 ± 0.3 µg/g lipid wt vs 0.1 ± 0.08 µg/g lipid wt; W = 187, $p = 0.004$) and marginally higher EROD activities (W = 146, $p = 0.04$). Sample sizes were small, but there was no difference in relative CYP1A content between males and females ($t_7 = 2.2$, $p = 0.063$), although males had a slightly lower average than females.

Both Catalina male and female stingrays had significantly lower contaminant concentrations compared with their mainland counterparts (W = 24, $p < 0.001$ and $t_{28} = -10.5$, $p < 0.001$, respectively; Figure 6A). In addition, proportions of the 3 contaminant groups were significantly different between the sexes by location (Supplemental Data, Figure S3). Both Catalina males and females had significantly higher proportions of PCB contaminants compared with mainland stingrays ($t_{25,12} = 2.9$–5.4, $p < 0.0001$). Mainland females had higher contributions of DDTs and chlordanes, but they were not significantly different from proportions measured in Catalina females, although chlordanes were nearly significant ($p = 0.91$ and 0.068, respectively). Catalina males, on the other hand, had significantly lower proportions of DDTs and chlordanes when compared with mainland males ($t_{11,40} = -2.3$ to 4.7, $p < 0.0001$–0.042).

Levels of EROD activity in male stingrays from the mainland (median, 615 pmol/min/mg protein; range, 104–1408 pmol/min/mg protein) were significantly greater than those in males from Catalina Island (median, 58 pmol/min/mg protein; range, 44–387 pmol/min/mg protein; W = 60.5, $p < 0.0001$; Figure 6B). Correspondingly, mainland male stingrays had significantly greater relative concentrations of CYP1A (Welch's $t = 2.2$–22.2, with only 10 out of 1000 combination comparisons yielding p values greater than 0.05; all others $p < 0.0001$–0.04, median $p = 0.002$; Supplemental Data, Figure S2).

In contrast, EROD activities from mainland (median, 272 pmol/min/mg protein; range, 60–1408 pmol/min/mg protein) and Catalina Island (median, 261 pmol/min/mg protein; range, 60–886 pmol/min/mg protein) females were comparable ($W = 897$, $p = 0.65$), as was their relative CYP1A content (Figure 6B). Lipid content in the livers of mainland males was much lower than that of Catalina Island males (mean ± SD, 32.6 ± 9.7% and 37.4 ± 14.6%, respectively; $W = 886$, $p = 0.01$); however, liver growth rate was not different between mainland and Catalina Island rays ($F_{1,129} = 1.1$, $p = 0.31$).

Southern California sediment contaminants

Throughout Southern California, PCB contaminants were found to be fairly widespread (Supplemental Data, Figure S4). Higher PCB concentrations appeared to be greater near metropolitan areas, such as those found in Santa Monica Bay, Los Angeles and Long Beach Harbors, Orange County, and San Diego. Of the 43 congeners measured, dioxin-like congeners comprised 15% of summed PCB concentrations in sediment throughout Southern California. Highest chlordane concentrations were found along the coast, in particular in the inlets of estuaries, harbors, or areas receiving urban runoff (i.e., river and ocean outlets; Supplemental Data, Figure S4). Around the Palos Verdes Peninsula DDTs were highly concentrated, with more than 100 metric tons being dumped (Supplemental Data, Figure S4). It appeared that DDT concentrations decreased in sediments sampled south of Palos Verdes but not in areas around San Diego. The major component of total DDT in Southern California sediment was 4,4′-DDE (69%), followed by 4,4′-DDD (13%), 2,4′-DDE (10%), 4,4′-DDT (5%), and 2,4′-DDD (3%). The PAH concentration in sediment was considerably greater relative to the other 3 contaminant groups (Supplemental Data, Figure S4) and particularly high near areas with high urban influence. Those PAHs known to induce dioxin-like responses (benzo[*a*]anthracene, benzo[*b*]fluoranthene, chrysene, indeno [1,2,3-*cd*]pyrene) composed 25% of measured PAHs [38].

DISCUSSION

Summed contaminant concentrations were found to significantly increase with size and age in mainland stingrays, with accumulation being influenced by both of these factors. In males, dramatic increases in contaminant concentrations were not observed until after maturity (approximately 15.0 cm disk width [28]), which suggests that either changes in environmental exposure or factors related to reproduction influence bioaccumulation rates between juvenile and adult size classes. One explanation for this rise in concentration could be increased dietary contaminant input with age. Babel [28] documented dietary shifts in round stingrays, with younger individuals consuming a higher proportion of small crustaceans and annelids compared with adults, where bivalves predominate in the diet. In addition, teleost fishes, which are located at higher trophic levels and would have higher contaminant concentrations than these other types of prey, were found only in the stomachs of larger stingrays ([28]; K. Lyons, personal observation) and could represent a substantial increase in contaminant input even if teleosts are not a consistent prey item.

Alternatively, differential habitat use by juvenile and adult males could alter the types of contaminants to which they are exposed [41]. Ontogenetic habitat shifts with age have been documented to occur in many elasmobranch species, where juveniles will recruit to areas primarily used by adults once a minimum size is reached [42,43]. If juvenile and adult male stingrays were utilizing similar habitats, their exposure to certain contaminant constituents should be the same. However, the increased proportion of PCBs and decrease in DDTs with size implies that adults and juveniles utilize different areas with varying contaminant compositions.

The differences observed among male size classes in their PCB profiles may also be attributed to variation in habitat use and dietary shifts with age. However, the distinct pattern of adults with greater proportions of higher chlorinated congeners compared with juveniles suggests that stingrays may have the ability to metabolize and excrete certain types of PCB congeners. Other vertebrates (e.g., mammals with greater metabolic activities than stingrays) have shown similar patterns, where older individuals have greater contributions from the higher chlorinated, more recalcitrant PCB contaminants that bioaccumulate with age because of their inability to be metabolized [39]. Interestingly, the most chlorinated congener group (deca) was higher in the smallest size class of stingrays and decreased with size in juveniles but subsequently increased in proportion in adults. This may be a result of maternal transfer during gestation, giving the youngest stingrays a higher than expected relative concentration of decachlorinated congeners than they would otherwise accumulate through their own feeding [44].

Factors related to reproduction also appear to play an important role in contaminant bioaccumulation, which affects not only accumulation rates between juvenile and adult size classes but also differences in sex between mature males and females of comparable ages. Bioaccumulation was not observed, nor did concentrations significantly increase, until after maturity for both sexes. Juvenile males and females, unlike adults, had comparable contaminant concentrations; and males showed a significant reduction with increased size until maturity (at approximately 15.0 cm disk width). Juvenile females showed similar trends, although marginally insignificant ($p = 0.051$–0.077), and stronger patterns may be present if a larger sample size was considered. This phenomenon has been observed in other species of elasmobranchs and could be attributed to juveniles growing at a faster rate than they acquire contaminants from the environment, resulting in a decrease in contaminant concentration with size until growth substantially slows [34].

After maturity, male and female stingrays showed marked differences in bioaccumulation rates as well as contaminant concentrations, with females having significantly lower levels than males. This trend has been demonstrated in a wide variety of marine mammal taxa [10,13] and is attributed to females' ability to depurate through maternal offloading processes [11]. Indeed, maternal offloading has been demonstrated in round stingrays [44] and is most likely 1 factor accounting for the contaminant differences between the sexes. Females were also found to have significantly larger livers and relatively higher concentrations of hepatic lipids than comparably sized males. Therefore, females' greater ability to dilute contaminants with growth may be another factor in addition to maternal offloading resulting in their significantly lower concentrations and bioaccumulation rate compared with males. Hepatic lipid concentrations in juvenile males increased with size, peaking just before maturity, and subsequently decreased in adult males. The decrease in hepatic lipids in adult males is likely a result of more energy shunted toward reproductive purposes and less devoted to storage [45–47]. The continual increase in liver weight but decrease in hepatic lipids with size would result in a concentrating effect of hepatic contaminants and may influence the large increase in bioaccumulation rate for adult males.

Besides differential contaminant bioaccumulation, male and female stingrays also showed differences in their contaminant profiles between juvenile and adult age classes. Mature females are known to sexually segregate from males after mating [28] and tend to aggregate in large numbers in calm embayments or estuaries with warm temperatures [48]. The dramatic increase in chlordane compounds and decrease in PCB proportions in mature females with age is likely reflective of their relatively higher use of these estuarine habitats for reproductive purposes, which are subjected to a high input of urban runoff during storm events [49] (Supplemental Data, Figure S4), compared with juveniles and males and Catalina female stingrays. However, the relatively low proportion of DDTs in both mainland male and female stingrays, despite their benthic lifestyle, indicates that stingrays are not utilizing areas near Palos Verdes shelf, where a large reservoir of DDT is still present in sediment [29]. Babel [28] noted an absence of stingrays caught in trawl surveys in the Santa Monica Bay area compared with areas south of Palos Verdes (i.e., Newport, Seal Beach, Bolsa Chica) and attributed this difference to unfavorable sediment composition. Stingrays sampled from Seal Beach probably utilize areas that are closer to shore and farther south at locations where the strong DDT signature associated with the Palos Verdes Superfund site are diminished relative to PCBs in the environment [29]. In addition, the significant difference between contaminant proportions for the 3 contaminant groups between Catalina and mainland stingrays further supports the hypothesis that contaminant profiles can be used to gain insights into habitat use. The significant increase in PCB contaminant proportion in livers of Catalina stingrays compared with mainland stingrays demonstrates PCBs' ubiquity throughout the environment and the attenuation of specific regional coastal signals (i.e., DDT and chlordane signals) as animals utilize more distant habitats.

Despite increasing concentrations of coplanar PCBs, females demonstrated a significant decrease in EROD activity with size, a trend not found in males. Given the relatively high plasma estradiol concentrations in pregnant female round stingrays at this time [50], the observed difference in biochemical response and relative CYP1A content between the sexes may be endocrine-related because estrogens are known to downregulate the expression of CYP1A enzymes [27]. However, the lack of relationship between EROD activity and coplanar PCB concentration suggests that other contaminants not measured in the present study, such as PAHs, may be influential. We did not measure PAHs in stingray livers because their high metabolic turnover led us to assume that any measurements taken from livers would not be reflective of actual concentrations present at exposure. High concentrations of PAHs, a substantial portion of which are AhR binding PAHs, were found in sediment sampled throughout Southern California, which highlights the substantial influence these densely urbanized contaminants have on the local marine environment. Certain PAHs can be strong inducers of the CYP1A system [51], and their high and ubiquitous presence throughout Southern California likely contributes greatly to the induction of the CYP1A system in stingrays. For example, round stingrays in the present study sampled from both a relatively clean and a contaminated area had substantially higher EROD activities compared with other elasmobranchs sampled from the East Coast, including those that had been treated with various CYP1A inducing agents [52,53] (Supplemental Data, Figure S5). Most of these species (little skate, *Raja erinacea*; large skate, *Raja ocellata*; dogfish, *Squalus acanthias*) utilize deep-water or open-ocean habitats that may be influenced substantially less by anthropogenic inputs into the local marine environment compared with the round stingray, which uses coastal habitat in a highly urbanized area.

In contrast to mainland rays, stingrays sampled from Santa Catalina Island did not show evidence of bioaccumulation, despite the inclusion of larger (and likely older) or comparably sized animals. Catalina Island males exhibited a significant negative linear relationship with summed contaminant concentrations with size, whereas females showed no trend. The lack of relationship for Catalina Island females suggests that they accumulate contaminants at rates equal to growth dilution and/or depuration through maternal offloading. Males, on the other hand, may be less exposed to contaminants than females because of differential habitat use as a result of sexual segregation, which is supported by differences in their contaminant proportions (Supplemental Data, Figure S3). Females were sampled at the end of a large embayment (Catalina Harbor) that experiences low tidal flushing and is subject to human activities such as boating and aquaculture that may increase contaminant exposure, particularly PAHs, compared with males that were sampled from an area with a completely exposed coast. Nevertheless, both males and females sampled from Catalina Island had significantly lower concentrations of summed contaminants compared with their mainland counterparts, which suggests that overall environmental exposure is significantly reduced at the offshore island. Catalina Island males had significantly higher hepatic lipid concentrations than mainland males, which would further contribute to the differential observed between males at these 2 locations. Higher hepatic lipid concentrations in Catalina Island males may be the result of different, higher-quality food sources or reduced competition on the island compared with the mainland.

In addition, stingrays showed significant differences in biochemical responses to contaminant exposure that were related to location by sex. Mainland male stingrays had significantly greater EROD activities than males from Catalina Island, which is likely a result of the increased exposure of contaminants that induce the CYP1A system on the mainland. Interestingly, females from both locations had similar EROD activities despite mainland females having significantly greater concentration of coplanar PCBs. The lack of difference between females from the mainland and Catalina Island for both EROD activity and CYP1A expression despite differences in contaminant concentrations compared with the stark differential between males is suggestive of an estrogenic downregulating effect. Among Catalina Island stingrays, females had both significantly higher concentrations of coplanar PCBs and correspondingly higher EROD activities than males, although the significance was not strong ($p = 0.04$) and there was little difference in CYP1A expression between them. In addition, males that were sampled from Catalina Harbor had substantially greater EROD activities than males sampled from Empire Landing, supporting the hypothesis that females were exposed to greater levels of environmental contaminants than males on the island.

In summary, the present study demonstrates that several factors influence the bioaccumulation of organic contaminants in round stingrays. While the magnitudes may be different, the patterns found in the present study are likely true for other elasmobranchs. Similar to patterns in other higher vertebrates, contaminant accumulation varies between the sexes and by age, with the greatest disparity found between the oldest males and females. Total concentration and contaminant proportions of accumulated contaminants were found to vary by location, suggesting that contaminant signatures may be used as an

alternative ecological tool to study differential habitat use among animals. The present study is one of the first to demonstrate that CYP1A activity and expression can be influenced by sex and contaminant exposure in an elasmobranch species. Therefore, future studies investigating contaminants and physiological response in elasmobranchs should take into consideration the potential influence that age, sex, and habitat use have on the study organism when interpreting results.

SUPPLEMENTAL DATA

Table S1.
Figures S1–S5. (7.9 MB DOCX).

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